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*EDITORIAL*

# THE IMMUNOLOGY OF IMMEDIATE AND DELAYED HYPERSENSITIVITY REACTION TO GLUTEN

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**The immunology of gluten hypersensitivity and celiac disease has been pursued with significant interest in the past 20 years. For the prevention of systemic diseases, most pathogens that gain entry into our bodies must be met with an effective immune response, yet in the gastrointestinal tract it is equally important that commensal bacteria and a diverse collection of dietary proteins and peptides be recognized without eliciting an active immune response or constant activation of the inflammatory pathway. This phenomenon of hyporesponsiveness to food antigens is known as oral tolerance. This oral tolerance to dietary antigens is maintained by three different mechanisms: anergy, cell deletion and immune suppression. However, in the presence of mechanical/chemical stressors and infections, this tolerance may break down, and gut associated lymphoid tissues (GALT) will react to different luminal antigens. The reaction of GALT to these antigens may lead to the production of pro-inflammatory cytokines, opening of tight junctions, entry of undigested antigens into the circulation, and the subsequent production of IgA, IgG, IgM and IgE antibodies in blood and secretory components. Like any other food hypersensitivity reaction, gluten sensitivity can be divided into immediate and delayed hypersensitivities. In this review an attempt is made first to differentiate immediate hypersensitivity to gliadin, mediated by IgE, from delayed hypersensitivity, which is mediated by IgA and IgG. Furthermore, we attempt to differentiate between gluten hypersensitivity with enteropathy (celiac disease) and gluten hypersensitivity without enteropathy.**

*The mechanism of oral tolerance to dietary proteins*

Although mucosal surfaces are exposed to many dietary proteins and infectious agents, the immune system normally will not react to these antigens (1-4). Unresponsiveness or tolerance to these antigens is maintained by three principal mechanisms: anergy or functional unresponsiveness; deletion through programmed cell death or apoptosis; and immune suppression by regulatory T cells (Tregs). This induction of immune suppression or anergy to gliadin is shown in Fig. 1.

Although HLA-DQ2 or HLA-DQ8, which are tissue types known to be associated with celiac disease, is found in roughly 30% of the western population, celiac sprue is encountered in 1 out of 50 carriers. Most carriers of these genes, like the rest of the population, harbor some form of immune protection through regulatory T-cells.

The regulatory T cells are divided into two major groups:

a. Natural Tregs, which act in a contact-dependent fashion, and express CD25 and transcription

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factor FOXP3;

b. Adoptive Treg Type 1 cells ($TR_1$), which function in a contact-independent manner and may or may not express CD25 and FOXP3. The $TR_1$ and $TH_3$ cells preferentially synthesize immunosuppressive cytokines IL-10 and TGF-β, respectively, in order to maintain homeostasis of responses to foreign antigens, including gliadin.

In the absence of tolerance, gut associated lymphoid tissues will react to luminal antigens, which may lead to the production of IgA and IgM antibodies, pro-inflammatory cytokines and subsequent inflammation and tissue damage or autoimmunity (5). Immediate and delayed hypersensitivity to gluten are characterized by IgE-mediated reaction or IgG, IgM, IgA plus T-cell reaction to gluten when tolerance to gluten is either not established properly or broken in these conditions (1-6).

A. *Immediate type hypersensitivity to gluten*

Like any other food hypersensitivity reaction, gluten hypersensitivity can be divided into immediate or delayed. The immediate hypersensitivity to gluten is IgE-mediated and may become life-threatening in severe cases when combined with exercise or some medication. This IgE-specific reaction may occur with IgE-specific epitopes of ω-5 gliadin, glutenins or allergenic epitopes of wheat formed after heat inactivation, hydrolyzation or chemical processes (6).

Food-dependent exercise or medication-induced anaphylaxis (FDEIA) is a distinct form of a common food allergy induced by a combination of causative food ingestion (wheat), physical exercise, and/or aspirin intake. Systemic allergic reactions such as anaphylactic shock and generalized urticaria are symptoms of FDEIA (7-8). This immediate hypersensitivity reaction is not limited solely to wheat antigen. Many kinds of foods such as shrimp, shellfish, hazelnut, buckwheat, corn, apple and orange have been reported to cause this type of food allergic reaction (9-14). The mechanism for food induction of IgE-mediated hypersensitivity is shown in Fig. 2.

Diagnosis of FDEIA is normally done by an exercise challenge test combined with ingestion of food that is known to have given patients episodes of anaphylaxis after its intake. The challenge test is unsafe for patients since it can provoke anaphylactic shock during testing. Therefore, an *in vitro* diagnostic method predicting development of symptoms by food and exercise challenge is a safer option for testing. However, for accurate *in vitro* testing it is necessary to identify IgE-binding epitopes (8).

This identification of IgE-binding epitopes of gliadin and high molecular weight gluten subunit was completed using sera from patients with WDEIA and enzyme immunoassay. Twenty-nine of thirty patients with wheat-dependent-exercise-induced anaphylaxis had specific IgE antibodies to these epitope peptides. Conversely, none of the 25 sera from healthy subjects reacted to both gluten and gluten peptides. These results indicate that measurement of IgE levels specific to epitope peptides of ω-5 gliadin and high molecular weight gluten peptide is useful as an *in vitro* diagnostic method for the assessment of patients with wheat-induced exercise-induced anaphylaxis, baker's asthma and contact urticaria.

In addition, in many industries wheat isolates have been produced by means of chemical and enzymatic treatment. This treatment induces the solubilization of gliadins in aqueous buffers by means of deamidation (15). The high protein content and solubility of treated gliadin in water provide interesting technological properties for their use in the food industry. The wheat isolates are used as food emulsifiers, gelling agents, film formation aid, stretchability agents in meat products, sauces, soups, and as clarifying agents in red wines.

This extensive use of wheat isolates in the food industry may be the major cause of hidden food allergies, which can be extremely dangerous to individuals with IgE-mediated allergy to wheat. In fact, anaphylaxis to wheat isolates was recently reported and proved by means of double-blind, placebo-controlled food challenge. Interestingly, the subject individual did not react to native wheat flour, but had very severe reaction to wheat antigens isolated from meat products. It was therefore concluded that treatments used for gluten deamidation generate new allergenic epitopes. A case of contact urticaria was recently attributed to hydrolyzed wheat in cosmetics combined with a generalized urticaria induced with

the ingestion of sausages with lentils and a French cassoulet. This patient could also eat cereal-based products without any problem (15-17).

Because food isolates or deamidated gluten are new food ingredients, when allergy to wheat is suspected, immune reaction to wheat isolates should be tested for a final determination of allergy to wheat or its chemically modified antigens.

*B. Delayed type hypersensitivity to gluten*

Delayed type hypersensitivity to gliadin is IgG, IgA or T-cell mediated. This reaction to gluten develops because of the loss or failure of the tolerance mechanism, or intolerance to ingested gluten. When this immune reaction to gluten occurs with the involvement of tissue transglutaminase in genetically susceptible individuals who present chronic inflammation in the small intestine, villous atrophy and flattening of the mucosa, it is called celiac disease. However, this immune reaction to gliadin and glutenin peptides of gluten may also occur in an individual without the involvement of genetic makeup and tissue transglutaminase, being induced instead by a loss of immune tolerance to gluten peptides and by enhanced gut permeability (18-19). If this loss of tolerance to gluten peptides does not involve enteropathy and is accompanied by intestinal barrier dysfunction, followed by the entry of these peptides into the circulation and systemic IgG and IgA response to gluten, then for this delayed type hypersensitivity we suggest the terminology gluten sensitivity without enteropathy.

*B1. Celiac disease or gluten sensitivity with enteropathy*

Celiac disease (CD) is a typical complex inflammatory disorder in which crucial genetic and environmental factors have been identified. It is an acquired disorder occurring in both adults and children. The condition is characterized by sensitivity to gluten that results in inflammation and atrophy of the mucosa of the small intestine. Similar protein components of related grains such as barley, rye, oat, kamut and spelt also cause an immune response in patients with CD. The clinical presentation of CD is very non-specific, and may vary from patient to patient. Patients may complain of abdominal cramps, bloating, diarrhea, and/or excessive gas production after meals. They may also note general malaise, lassitude, weakness, undesired weight loss, constipation, anemia ($B_{12}$ deficiency), osteoporosis/osteopenia, poor dentition, peripheral neuropathy, seizures/ataxia with cerebral calcifications, irritability or poor growth in children, birth defects in infants, small stature, and amenorrhea/infertility/recurrent miscarriage in females (18-21).

*Diagnosis of celiac disease*

Because CD presentation varies so greatly, many affected individuals do not suspect they have the disease and therefore do not seek medical attention. Even when medical attention is sought, if patients have atypical symptoms, CD may not be diagnosed unless the physician suspects and tests for it. Therefore, diagnosed celiac disease is most likely the 'tip of the iceberg' accounting for only approximately 12% of total cases. Characteristic villous atrophy and symptoms of intestinal malabsorption are present in the classic form of the disease (22); however, now many newly-diagnosed patients have milder, atypical symptoms often without diarrhea or malabsorption ("atypical CD") or have no symptoms at all ("silent CD").

Recently, serological testing has been increasingly used to test patients with suspected gluten-sensitive enteropathy as well as for monitoring dietary compliance. Both IgG and IgA antibodies are detected in sera of patients with gluten-sensitive enteropathy (5). IgA antibodies are less sensitive but more specific markers of the disease. IgG antibodies appear to be more sensitive but less specific markers of disease than IgA. It is recommended that both antibodies should be measured due to the high incidence of IgA deficiency among celiac patients, which may mask the disease. Antibody testing is also important in detecting individuals who are at risk of having celiac disease but have no symptomology, in individuals with atypical symptoms or extraintestinal manifestations of celiac disease (gluten sensitivity without enteropathy), and in individuals with presumed celiac disease who fail to respond to a gluten-free diet. Patients with positive antibody tests must undergo small intestine biopsy to confirm the diagnosis and assess the degree of mucosal involvement (23-25).

*Immune mechanism in celiac disease*

Gluten is composed of two proteins, gliadin and glutenin. Gliadin, the alcohol-soluble component, is the preferred substrate of tissue transglutaminase, an enzyme that deamidates or removes an amino group from gliadin and adds the remainder of the peptide into existing proteins as part of the normal repair process. Transglutaminase is present in the cytoplasm of most cells in an inactive state, but inflammation and mechanical injury activate and release it into the intracellular matrix. It is present in high concentrations in the connective tissue of the small intestinal wall, especially surrounding smooth muscle cells in the lamina propria. Transglutaminase complexes with gliadin to form a "neoantigen" recognized as immunogenic by patients with celiac disease. The neoantigen is processed by antigen-presenting cells such as macrophages, which then present it to CD4+ T-lymphocytes. The CD4+ T-lymphocytes then activate to produce interferon-γ and to proliferate. Interferon-γ, produced by T cells, is thought to be primarily responsible for injuring and killing mucosal epithelial cells (19-20). This immunological mechanism underlying celiac disease in individuals with specific HLA subtype is shown in Fig. 3.

In addition to mechanical stress, chemical injury, infectious agents, macrophages and CD4+ T-lymphocytes, other lymphocyte subsets are also involved in the immune response in CD. Early in celiac disease, certain "toxic" small gliadin peptides generated by transglutaminase activity stimulate secretion of IL-15 by epithelial cells and lamina propria macrophages. These gliadin peptides also increase mucosal permeability, enhancing lymphocyte infiltration. IL-15 is a key inflammatory mediator that stimulates intraepithelial lymphocytes. The humoral immune mechanism is activated when sensitized CD4+ T cells stimulate B cells to make anti-gliadin and anti-transglutaminase antibodies. B-lymphocytes mature into increased numbers of plasma cells in the intestinal submucosa where they produce the antibodies characteristic of CD. The presence of T cells that recognize deamidated gluten peptides in celiac disease might be relevant to autoimmunity and the implication of celiac disease in many autoimmune diseases (26-29).

*C. Delayed hypersensitivity to gluten without enteropathy or gluten sensitivity without enteropathy*

The terms gluten sensitivity and celiac disease (also known as gluten-sensitive enteropathy) have thus far been used synonymously to refer to a disease process affecting the small bowel and characterized by malabsorption and gastrointestinal symptoms. Yet, gluten sensitivity can exist even in the absence of an enteropathy. The systemic nature of this disease, the overwhelming evidence of an immune pathogenesis and the accumulating evidence of diverse manifestations involving organs other than the gut, such as the skin, heart, bone, pancreas, joints, nervous system, liver, uterus and other organs necessitate a re-evaluation of the belief that gluten sensitivity is solely a disease of the gut (30). This involvement of multi-organ system disorder could be independent of HLA type and production of antibodies against tissue transglutaminase (26, 30). The immune reaction to gliadin peptide and its cross-reaction with different tissues might result from a breach in oral tolerance to gliadin and the induction of intestinal barrier dysfunction by environmental factors such as xenobiotics and infections (rotaviruses).

Indeed, human rotaviruses are the most frequent etiologic agents of gastroenteritis in infants and young children in most parts of the world. Anti-gliadin peptide antibodies from patients with gluten sensitivity recognize the viral product, suggesting a possible link between rotavirus infection and gluten sensitivity. It has also been demonstrated that purified rotavirus peptide antibodies are capable of cross-reacting with gliadin peptide, tight junction protein (desmoglein peptide) and monocytes toll-like receptor-4 peptide. These findings further implicate alteration of cell permeability in gluten sensitivity and autoimmunity (26, 31-32).

Therefore, since affinity-purified rotavirus peptide antibody not only binds to gliadin peptide but also recognizes endomysial structure, activates TLR4, and alters epithelial cell permeability, it suggests that the rotavirus epitope may be important in determining an anti-virus immune response's ability to cross-react with self-antigens. This cross-reaction between rotavirus peptide and human tissue antigens has functional consequences on TLR4, tight junction proteins and intestinal permeability. It

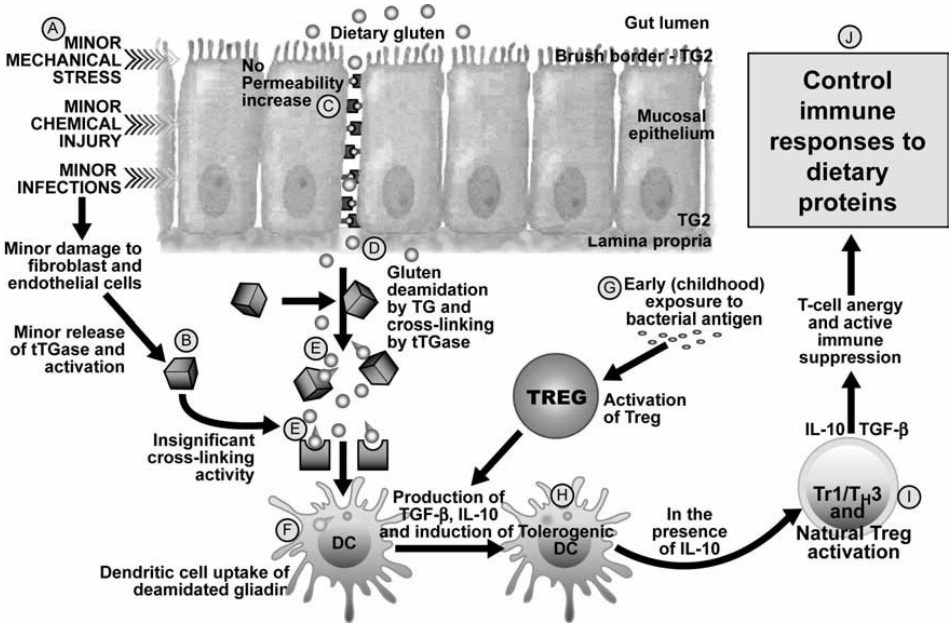


**Fig. 1.** *Cellular and molecular induction of immune tolerance to dietary proteins (gliadin). In the absence of major mechanical and chemical stress or infection (A), no damage is done to fibroblasts and epithelial cells, and only small quantities of tissue transglutaminase are released into the environment (B). Since under these conditions the tight junctions are in perfect shape (C), only a few gliadin molecules may survive digestion and be transported across the mucosal epithelium (D). If these molecules of gliadin are deamidated by transglutaminase (E), the key regulator of the immune system called dendritic cells or antigen-presenting cells (F) prime T cells for anergy or tolerance. Early exposure to dietary proteins and bacterial antigens such as LPS (G) can activate regulatory T cells to produce TGF-β and IL-10, inducing activation of tolerogenic DCs (H) to control immune response to dietary proteins (gliadin). Further activation of $TR_1$, $TH_3$ and natural Treg (I) by IL-10 results in induction of central or peripheral tolerance (J).*

is likely, then, that a molecular mimicry mechanism may be involved in the pathogenesis of gluten sensitivity with or without enteropathy (33-37).

The gliadin peptide also shares homology with other self-antigens such as heat shock protein-60 (HSP60), glutamic acid decarboxylase, myotubularin-related protein-2 and toll like receptors. Heat shock proteins are highly conserved proteins synthesized when cells are exposed to stress stimuli, such as infection and inflammation. Increased expression of HSPs has been observed in jejunal epithelial cells in patients with CD. Antibodies against the celiac peptide cross-react with HSP60 and may therefore induce epithelial cell cytotoxicity, thus amplifying the damage of the intestinal mucosa with increased intestinal permeability (37).

Matrix metalloproteinase-2 (MTMR2) belongs to the protein-tryrosine phosphatase family. Defects in MTMR2 are the cause of Charcot-Marie-Tooth disease type 4, which is an autosomal recessive demyelinating neuropathy. A demyelinating nervous system disease can be observed in patients with CD.

Finally, TLRs are type I transmembrane proteins involved in innate immunity by recognition of conserved microbial structures. Activation of antigen presenting cells via innate immune receptors such as TLR4 can break self-tolerance and trigger the development of autoimmunity (38-40). The anti-gliadin peptide antibodies bind TLR4 on monocytes and induce both the expression of activation molecules such as CD83 and CD40 and the production of pro-inflammatory cytokines similar to the action of bacterial antigens. The mimicry mechanism by which rotaviruses or other environmental factors are involved in the pathogenesis of gluten sensitivity without enteropathy is shown in Fig. 4.

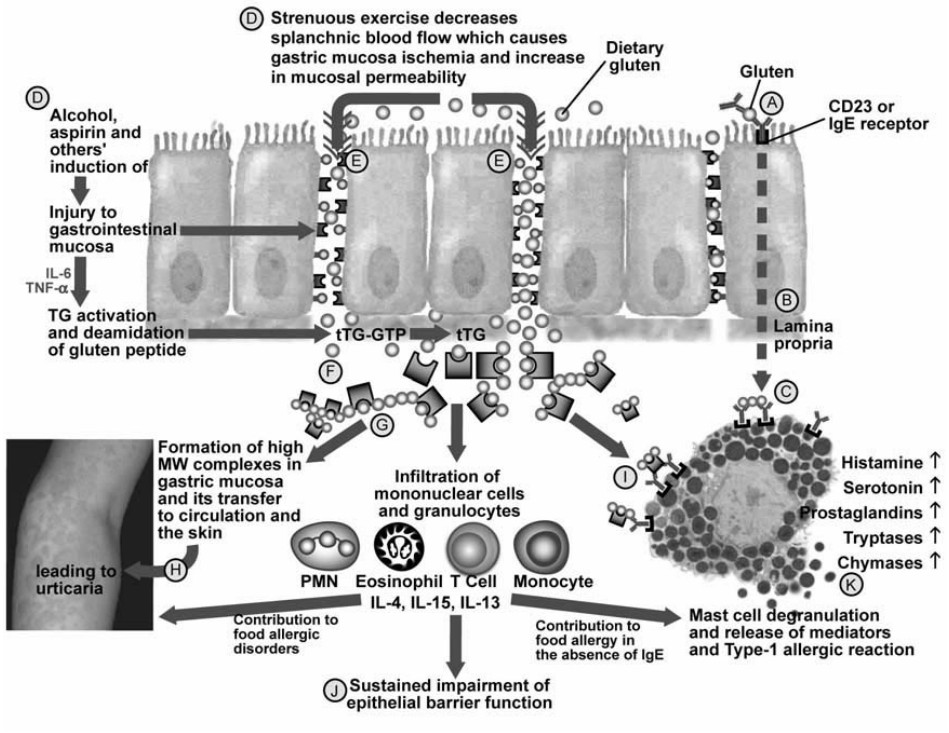


**Fig. 2.** *Schematic presentation of the pathophysiology of the immediate hypersensitivity reactions (Type I allergy) of the intestine. Hypersensitivity reaction occurs by the binding of dietary peptides (gluten) to low affinity IgE receptor CD23, which is expressed on the epithelium of the small intestine (A), facilitating uptake of antigen in an IgE-independent manner (B). Gluten cross-links to IgE on the surface of MAST cells to induce degranulation (C). This MAST cell degranulation could be induced by strenuous exercise, alcohol and medication (aspirin) (D), causing injury to gastrointestinal mucosa and an increase in mucosal permeability (E). Under these conditions, parts of gluten that are resistant to processing by luminal and brush-border enzymes will survive digestion and be transported across the mucosal epithelium as polypeptides. Upon activation of transglutaminase in the subepithelial region (F), many gliadin peptides form high molecular weight complexes with transglutaminase (G) that can be transferred into the circulation and the skin, leading to urticaria (H). These complexes can also bind to IgE receptors on MAST cells and induce further degranulation (I). Finally, infiltration of granulocytes, mononuclear cells and their cytokines can contribute to late phase responses, which result in the impairment of epithelial barrier function (J). Also, products released from MAST cells, including histamine, serotonin, prostaglandins, tryptases and chymases (K), have been shown to have direct and indirect effects (via activation of the enteric nerve) on epithelial ion secretion, barrier function, and intestinal motility.*

Based on this mechanism of action, we should think about the immunology of gluten sensitivity beyond the gut and emphasize laboratory testing for celiac disease and gluten sensitivity beyond gliadin and transglutaminase antibodies.

## CONCLUSIONS

Immediate type hypersensitivity to gluten is IgE mediated. This IgE-mediated reaction to gluten may become life-threatening if wheat ingestion is combined with exercise or with medication, such as aspirin.

Strenuous exercise, medications and xenobiotics, by decreasing splanchnic blood flow, may cause an increase in mucosal permeability and the entry of gliadin into the circulation, hence, antibody response against gliadin polypeptides.

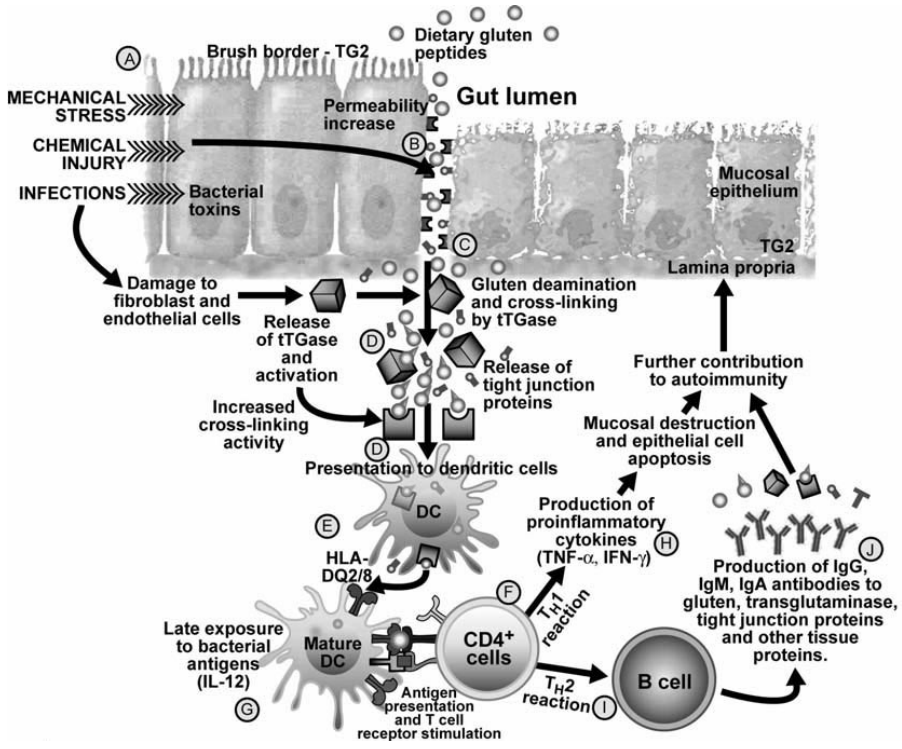


**Fig. 3.** *Depiction of the intestinal mucosa with emphasis on the factors involved in the development of celiac disease in individuals with HLA-DQ2/DQ8 positive. Infection, mechanical and chemical stress (A) can impair mucosal integrity (B). The parts of gluten that are resistant to brush-border enzymes will survive digestion and can be transported across the epithelial barrier as polypeptides (C). Tissue transglutaminase in the intestinal mucosa (lamina propria) become activated and deamidate gluten peptides. Some of the deamidated gliadins may cross-link to transglutaminase and form complexes of gliadin with tTG (D). Deamidated gliadin peptide by itself, deamidated gliadin peptide cross-linked to tTG, and released tight junction proteins are presented by dendritic cells or antigen-presenting cells as well as B cells (E) which carry HLA-DQ2 or DQ8 molecules to the CD4+ T cells in the lamina propria (F). It is believed that this antigenic presentation is enhanced in an individual with later-in-life exposure to bacterial antigens whose mature dendritic cells produce significant amounts of interleukin-12 (G). This antigenic presentation results in driving the CD4+ cell response either towards $T_H1$ reaction, production of inflammatory cytokines (H), mucosal cell destruction and autoimmunity, or, toward $T_H2$ response B-cell activation (I), and antibody production against deamidated gluten, transglutaminase, gliadin cross-linked to transglutaminase, and different tissue antigens (J).* - *Deamidated gliadin peptide;* - *deamidated gliadin peptide cross-linked to tTG;* - *tight junction proteins;* - *transglutaminase;* **T** - *different tissue antigens.*

Clinicians should be aware that during food processing many wheat isolates are produced by chemical and enzymatic treatment and used in many food products. Therefore, some patients may have immune reaction to treated gliadin used in sausage, but not to gluten or wheat itself.

Unlike immediate type hypersensitivity to gluten, which occurs within minutes, the delayed type hypersensitivity to gluten may occur hours or days after ingestion of wheat.

Delayed type hypersensitivity to gluten is an antibody- (IgG, IgA) and T-cell-mediated reaction. Immune reaction to gluten occurs in genetically susceptible individuals with the involvement of tissue transglutaminase, resulting in chronic inflammation of the small intestine. This delayed type hypersensitivity to gluten is called celiac disease or gluten sensitivity with enteropathy.

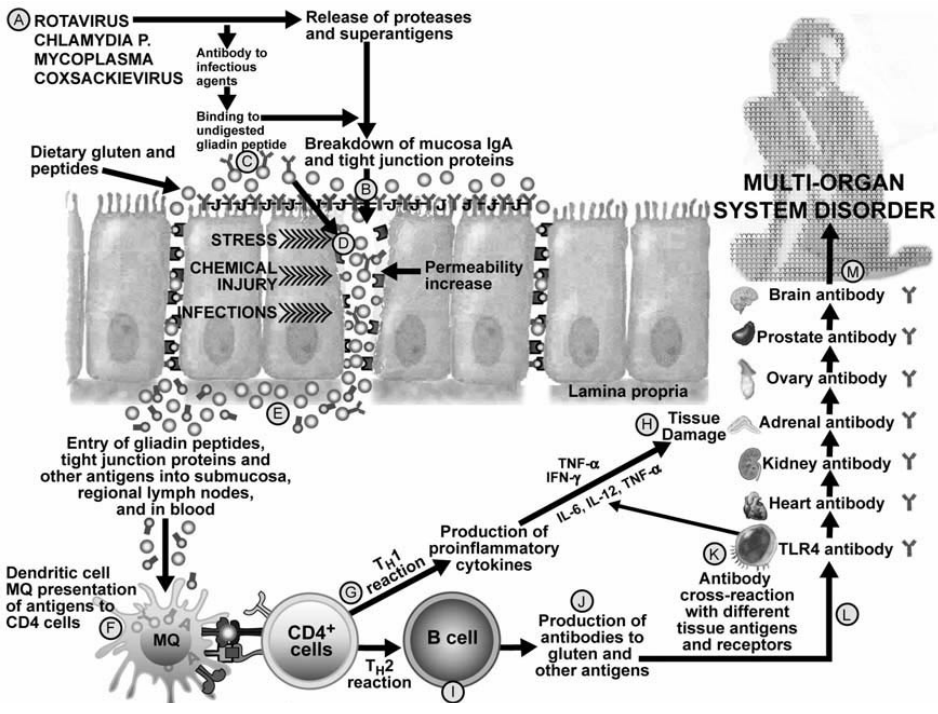


**Fig. 4.** *Depiction of immunological mechanisms underlying gluten sensitivity and its immunopathological consequences. Precipitation of gluten sensitivity without enteropathy appears to be preceded by acute gastroenteritis symptoms induced by infections such as rotavirus and others (A). Rotavirus and its super-antigens can break down mucosal IgA directly (B) or indirectly by the local production of anti-rotavirus antibody. Due to partial linear homology or cross-reactivity between rotavirus protein and a-gliadin, the anti-rotavirus antibody binds to gliadin and forms complexes with it (C). The combination of infection antibody cross-reactivity with gliadin and additional stressors can severely impair mucosal integrity (D) and the entry of gliadin peptides, tight junction proteins and other antigens into the submucosa, regional lymph nodes, and the blood (E). Gliadin peptides, rotavirus antigens, rotavirus antibody bound to gliadin, and tight junction proteins are presented by dendritic cells with or without HLA-DQ2/DQ8 to CD4+ cells (F). This antigenic presentation results in driving the cell CD4+ response either towards TH1 reaction (G), the production of proinflammatory cytokines, which contributes to autoimmunity (H); or towards $T_H2$ response B-cell activation (I) and antibody production against gluten, rotavirus, and tight junction proteins (J). Cross-reaction of these antibodies with cell receptors such as toll-like receptors on monocytes and the release of IL-6, IL-12 and TNF-γ (K), and tissue antigens such as heart, kidney, adrenal gland, ovary, prostate, brain and others (L) results in further tissue damage and multi-organ system disorders (M).* - *Gliadin peptides;* - *rotavirus antibody bound to gliadin;* - *tight junction proteins.*

Gluten sensitivity without enteropathy may occur in individuals without the involvement of genes, tissue transglutaminase and presence of inflammation in the small intestine. Gluten sensitivity without enteropathy is induced mainly by enhanced gut permeability due to infection (rotavirus), stress or chemical injuries.

Impaired mucosal integrity results in the entry of gliadin peptides, tight junction proteins and others to the submucosa, regional lymph nodes, and the blood. The entry of gliadin peptides, tight junction proteins and infections in the blood results in the production of antibodies against them.

The cross-reaction of these antibodies with different tissue antigens such as heart, kidney, adrenal gland, ovary, thyroid, parathyroid, prostate,

brain and others results in multi-organ disorder, which will be discussed in a subsequent article.

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*EDITORIAL*

# CHOLESTASIS AND PREGNANCY

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**Obstetric cholestasis is an intrahepatic multifactorial disease, unique to pregnancy which presents with intense pruritis and abnormal liver function tests (LFTs). It commonly presents in the third trimester and becomes more severe with advanced gestation. The prevalence of obstetric cholestasis is influenced by genetic and environmental aspects and varies in different populations. The pathogenesis appears to relate to a predisposition to the cholestatic effect of increased circulating oestrogens and progestogens. Also genetic mutations have been reported in a sub-group of women with elevated γ-GT. There can be significant maternal morbidity due to intense pruritis and consequent sleep deprivation. There may be malabsorption with steatorrhea resulting in vitamin K deficiency, prolongation of clotting times and increased risk of postpartum haemorrhage (PPH). Caesarian section rate is much higher for women with obstetric cholestasis. The potential fetal risks include preterm labour and prematurity and unexplained intrauterine death. The aim of the management of obstetric cholestasis is to avoid fetal complications and to relieve maternal symptoms. A variety of drug therapies have been used to reduce maternal pruritis. Policies of active management and induction of labor before 38 weeks may improve pregnancy outcome. Obstetric cholestasis is a diagnosis of exclusion, and other causes of pruritis should be excluded.**

Obstetric cholestasis is an intrahepatic multifactorial disease, unique to pregnancy which presents with intense pruritis and abnormal liver function tests (LFTs) (1). It is an important condition to diagnose because of the adverse effects in pregnancy with which it is associated. The potential fetal risks include preterm labor and prematurity and. Rarely, intrauterine death (1-2). There can also be significant maternal morbidity due to intense pruritis and consequent sleep deprivation (1). Accurate differentiation from those women who have itching in pregnancy without the disease is often difficult. Most authorities accept elevations of any of a wide range of LFTs beyond pregnancy-specific limits as pathognomonic for obstetric cholestasis as long as other causes of abnormal blood results are accurately excluded (1, 3-4). While medical treatments have not been conclusively shown to be of benefit, policies of active management may improve pregnancy outcome (3, 5).

The prevalence of obstetric cholestasis is influenced by genetic and environmental aspects and varies in different populations, with reported rates varying from 0.2% in France to 0.7% in England, 1% in Finland, 1.5% in Poland and Spain and 12% in Chile (1-2, 6). The pathogenesis appears to relate to a predisposition to the cholestatic effect of increased circulating oestrogens and progestogens (7). Also genetic mutations have been reported in a subgroup of women with elevated γ-GT (8). Women may

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report a positive family history, cyclical pruritis, or pruritis when taking the combined oral contraceptive pill (9).

This paper aims to review the literature in order to facilitate the clinician to accurately diagnose and treat the disease.

*Maternal morbidity and postnatal course in obstetric cholestasis*

There can be significant maternal morbidity due to intense pruritis and consequent sleep deprivation which could last for several weeks until the decision for delivery is taken. There may be malabsorption with steatorrhea resulting in vitamin K deficiency, prolongation of clotting times and increased risk of postpartum haemorrhage (PPH). Caesarian section rate is much higher for women with obstetric cholestasis, ranging from 10% to 36%. Reasons why the rate is higher could be induction of labor, obstetric problems, clinical course of cholestasis and maternal/obstetrician anxiety (7, 9).

Because of obstetric cholestasis, both pruritis and increased LFTs remit following delivery. Pruritis is usually absent within 48 hours following delivery (3). A prospective study of 91 women with obstetric cholestasis reported a maximum rise of bilirubin at days one or two and for AST, ALT at days two and five (3). In normal pregnancies, LFTs may increase the first 10 days of the puerperium, but in cholestasis are increased beyond this period (10-11). Therefore, in pregnancies complicated with obstetric cholestasis, routine measurements of LFTs should be deferred beyond this time. In the majority of cases liver function tests will be normal in 7-10 but in some cases it might take up to 4 to 6 weeks postnatally (9-10). The disease tends to recur in subsequent pregnancies (7). Some women may report pruritis associated with the use of combined contraceptive pills. In addition, gallstones are more common in women with a history of obstetric cholestasis.

*Clinical presentation and diagnosis of obstetric cholestasis*

The classical clinical feature of obstetric cholestasis is generalized severe pruritis which most commonly presents in the third trimester and becomes more severe with advanced gestation (1). The pruritis is often severe and typically worse at night. It is often widespread, usually involving the palms of the hands, the soles of the feet and the trunk (5). Women often report history of pruritis of several weeks. Additional symptoms which may be reported are right upper quadrant pain, pale stools and dark urine (9). If jaundice develops, it tends to follow the pruritis by 2-4 weeks and to reach a plateau relatively quickly.

In obstetric cholestasis, the most commonly elevated liver function tests are total serum bile acids and transaminases (mainly ALT) (9). Typically, transaminases will range from just above the upper limit of normal to several hundreds. Postnatal resolution of symptoms and of biochemical abnormalities is required to secure the diagnosis (1). Less commonly, bilirubin and γ-GT are raised in approximately 20% of cases. The serum total bile acid concentration is increased, and this is largely due to primary bile acids (7). Despite the elevated transaminases and bile acids, liver failure does not occur (3). Bilirubin is only raised infrequently, and most women will have increased levels of one or more of the remaining three LFTs. In a prospective Scandinavian study among women with obstetric cholestasis, 100% of women had elevated ALT, 99% elevated AST and 22% elevated bilirudin; 92% of the cases had elevated serum bile acids (3). Most experts would consider elevations in one or more of ALT, AST, γ-GT or total bile acids to be consistent with the diagnosis of obstetric cholestasis. Most recent studies included GGT in assessment of liver function and suggested that it is elevated in 40% to 50% of obstetric cholestasis cases (3). It is reported that the onset of the disease is earlier in carriers of hepatitis C (12).

Obstetric cholestasis is a diagnosis of exclusion, and other causes of cholestasis should be excluded. Possible causes of pruritis and abnormal liver function include hepatitis A, B, C, Epstein Barr, cytomegalovirus, liver autoimmune pathology, gallstones, biliary cirrhosis, hyperemesis gravidarum, pre-eclampsia and acute fatty liver of pregnancy (1, 3).

*Fetal risks in pregnancies complicated by cholestasis*

Obstetric cholestasis causes fetal distress (defined as either meconium staining of amniotic fluid or fetal

heart rate abnormalities), spontaneous preterm labour and unexplained third-trimester intrauterine death (1, 3). The risk of stillbirth increases towards term and does not correlate with the severity of maternal symptoms. Since 1965, all the evidence on outcome in obstetric cholestasis comes from case series and case control studies totaling 1,578 patients. Increased incidence of fetal distress (2-33%) and premature delivery (15.7-30%) have been linked with obstetric cholestasis (5, 7, 13-14). Meconium stained liquoir has been observed in 16-58% of cases (15). Actually meconium stained liquor is more frequently observed in preterm cholestasis than term cholestasis pregnancies (25% versus 12%) (1). The prediction and prevention of intrauterine death in obstetric cholestasis still remains a challenge. No specific fetal monitoring protocol has been recommended for the prediction of fetal demise. Intrauterine deaths have been reported in pregnancies with normal CTGs and umbilical artery Doppler investigations in the last five days (16-17). Other studies showed no correlation between fetal distress (abnormal CTG) and bile acid levels (1, 3, 13, 15). In addition, no correlation was found between umbilical artery Doppler studies and bile acid levels (1, 3, 13, 15).

The underlying cause for all these complications remains unclear. It is possible that high concentrations of fetal bile acids may contribute to an acute event resulting in fetal demise. A study in 2004 reported that levels of bile salts four times higher than normal limits were linked with similar increased risk of premature delivery and meconium stained liquor when compared with women with pruritis who had normal bile salts levels (14). Vasospasm due to cholic acid at the placental chorionic surface may lead to fetal hypoxia (18). Bile acids are known to have cellular effects that could mediate increased uterine contractility (19). As for the meconium staining, the suggested mechanism is that the bile acids have an effect on colonic musculature and subsequent motility (20).

*Treatment measures for obstetric cholestasis*

The aim of the management of obstetric cholestasis is to avoid fetal complications and to relieve maternal symptoms. Widely adopted policies of active management may indeed improve pregnancy outcome (3, 5). The first extensive study which suggested active management for cases of obstetric cholestasis was performed by Fisk (21). In this study a perinatal mortality of 107 per 1,000 was noted from 1965 to 1975, and in the same hospital from 1975 to 1984 the perinatal mortality dropped to 35 per 1,000 when active management was installed. The usual practice is to offer a delivery at 37 weeks, or at diagnosis if pregnancy is beyond 37 weeks (1, 3, 22). It would seem appropriate to offer all women with obstetric cholestasis the same policy of active management (intensive fetal surveillance antenatelly, elective early delivery before 38 weeks). On the other hand, possible consequences of higher rates of intervention (e.g. Caesarian section, iatrogenic prematurity, postpartum haemorrage) should be taken into account individually.

A variety of drug therapies have been used to reduce maternal pruritis. Topical solutions and creams (like diprobase, calamine lotion, aqueous cream with methanol) are widely used as a first line together with oral antihistamines, however not always with satisfactory results for the relief of symptoms. S-Adenosyl methionine is a second line treatment offered in many centers. There insufficient evidence, however, and the reports are conflicting as to the benefit of this medication either to control maternal symptoms or for improving the fetal outcome (1, 22-23). Its proposed administration regime is intravenous infusion of 600-900 mg intravenously. Ursodeoxycholic acid is a hydrophilic bile acid, which acts by altering the bile acid pool and in this way reduces the amount of hydrophobic, hepatotoxic bile acids. The proposed treatment dose is 1000mg /day for a period of 7 to 10 days. Evidence shows significant reduction in symptoms and LFTs (3, 23). There is insufficient data to support the widespread use of ursodeoxycholic acid against stillbirth and safety of the fetus and neonate (22, 24-25). It is reasonable to offer a daily supplement of vitamin K to pregnant women from the diagnosis of obstetric cholestasis. Maternal vitamin K administration in soluble form (10 mg once daily) reduces the risk of postpartum hemorrhage and fetal bleeding (5, 7). The rationale to the prophylactic administration of vitamin K is that obstetric cholestasis could lead to mal adsorption and subsequent deficiency of vitamin K and reduced formation of clotting factors II, VII, IX and X from

the liver. As for the use of dexamethasone, results are conflicting on the relief of symptoms, reduction of LFTs levels, and there is also deep concern about the possible adverse neonatal neurological effects (1, 3, 25). The usual dosage is 12 mg daily for the first week, then reducing the dose for three days.

As for the decision of delivery, there is accumulating evidence that the perinatal mortality rate is considerably reduced to 2.0-3.5%, for women who are delivered no later than 38 weeks gestation (1, 13-14).

## CONCLUSION

Obstetric cholestasis is a liver disease unique to pregnancy which presents with pruritis and abnormal liver function tests. It is important to accurately differentiate other pathologies with a similar clinical course, since obstetric cholestasis could lead to major complications and fetal death. Different medical treatments have been proposed and adopted with inconclusive benefits for maternal morbidity and fetal wellbeing. Active management may improve the outcome. Future research is needed in order to explore the multi-factorial and genetic background of the disease in order to enable to identify earlier those fetuses at risk.

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# DIFFUSION OF TWO POTENTIAL ANTI-HIV MICROBICIDES ACROSS INTACT AND DE-EPITHELIALISED, HUMAN VAGINAL MUCOSA

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**The lectins derived from *Hippeastrum* hybrid (*Hippeastrum* hybrid agglutinin, HHA) and from the stinging nettle *Urtica dioica* (UDA) have been investigated as anti-HIV microbicides. The present study was conducted to determine their diffusion through intact and de-epithelialised human vaginal epithelium. Both lectins were labelled with fluorescein isothiocyanate groups (FITC) and analysed by SDS-polyacrylamide gel electrophoresis. While UDA appeared to be fairly homogeneous with an average Mw of ~ 8.5 kDa, HHA was a heterogeneous mixture of compounds with Mw's ranging between ~13 and 52 kDa. Fresh human vaginal mucosa was snap-frozen in liquid nitrogen and stored at -85ºC. Prior to an experiment, the tissue was defrosted to 20ºC in PBS buffer, pH 7.4, and placed in the seven flow cells of a flow-through perfusion apparatus. Either FITC-labelled HHA or UDA was then pipetted into the donor chamber of the flow cell. Samples from each flow cell were collected every 2 hours (1.5 ml/h) over a 24-hour period and analysed by fluorospectrophotometry. Permeability experiments were repeated with vaginal mucosa specimens from which the epithelial layers had been removed by heat-stripping. Both lectins diffused through vaginal mucosa at rates proportionate to their average molecular weights, the flux rates of the smaller UDA being ~ 5x higher than that of the larger HHA. Removal of the vaginal epithelium increased the flux rates of both HHA and UDA across the mucosa and this may have implications for a more rapid *in vivo* uptake of these lectins when used as anti-HIV microbicides.**

Carbohydrate-binding agents (CBA) possess the potential to bind to the glycosylated envelope of the human immunodeficiency virus (HIV), and it has therefore been suggested that they may show promise as intravaginally applied anti-HIV microbicides (1). To date, a number of prokaryotic, fungal, invertebrate and plant lectins, with specificity for mannose and/or *N*-acetylglucosamine, have been studied with respect to their anti-HIV properties (2-3). Two of these agents, the mannose-specific lectin derived from *Hippeastrum* hybrid (*Hippeastrum* hybrid agglutinin, HHA) and the *N*-acetylglucosamine-specific lectin derived from the stinging nettle *Urtica dioica* (UDA) have been fairly thoroughly investigated as potential anti-HIV microbicide candidate agents (1). HHA has been described as a tetramer consisting of 4 x 12.5 kDa non-covalently linked subunits, each subunit possessing 3 carbohydrate binding sites and 3 cysteine residues. Two of these cysteine residues form an internal disulfide bridge, while the third is

*Key words: permeability, lectins, vaginal mucosa*

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free. HHA is not glycosylated. UDA on the other hand is a small monomeric protein (8.5 kDa) built up from two in-tandem hevein domains, each having 4 disulfide bridges. Both hevein domains are similar but not identical. Each domain has one carbohydrate binding site with different binding specificity for *N*-acetylglucosamine oligomers.

Most of the data obtained on HHA and UDA with respect to their mode of action, mutational pathways, resistance profiles and side-effects have been obtained by *in vitro* studies on HIV isolates. However, because of the interesting physiochemical characteristics of both these compounds, considerable challenges will be presented to pharmaceutical scientists for their formulation as topical vaginal anti-HIV microbicides. More data is therefore needed regarding their physicochemical stability, interactions with mucosal surfaces as well as inherent immunogenic and toxic properties. Because HHA and UDA have the potential, as anti-HIV agents, for being absorbed when topically administered to mucous membranes, the objective of the present study is to investigate the diffusion kinetics of HHA and UDA through intact and de-epithelialised human vaginal mucosa.

## MATERIALS AND METHODS

*Human vaginal mucosa*

Tissue specimens were obtained from excess tissue removed from five postmenopausal women, ages 49-70 y (mean age: 59 ± 9 y) following vaginal hysterectomies at the Louis Leipoldt Hospital, Bellville, South Africa.

All tissue specimens were immediately placed in a transport fluid and transferred to our laboratory within 1 h. The transport fluid consisted of a stock solution of Eagle's Minimum Essential Medium (MEM) without L-glutamine and sodium bicarbonate (Gibco, Paisley, Scotland), to which the latter as well as an antibiotic and antimycotic were added prior to using it for the transport of mucosal specimens. Excess connective and adipose tissue were trimmed away and all specimens were snap-frozen in liquid nitrogen and stored at -85°C for periods up to 6 months, as previously prescribed (4-8). No specimens were obtained where there was clinical evidence of any disease that might have influenced the permeability characteristics of the different specimens. The Ethics Committee of Stellenbosch University and the Tygerberg Academic Hospital approved the study.

*HHA and UDA*

The purified lectins HHA and UDA were kindly provided by Els van Damme and Willy Peumans (Ghent University, Ghent, Belgium) (2-3).

*Fluorescein isothiocyanate (FITC) labelling of HHA and UDA*

A FluoroTag FITC Conjugation Kit of the Sigma Chemical Co. (St. Louis, MO) was used. Fifty milligrams of both HHA and UDA was conjugated with FITC, using a 1:1 molar ratio. The reaction was carried out for 2 hours at room temperature in a 0.1 M carbonate-bicarbonate buffer, pH 9.0. In order to remove free fluorescein from the FITC-protein conjugates, the reaction mixtures were extensively dialysed against distilled water (HHA with Spectrapor tubing molecular weight cut-off 14 kDa and UDA with Snakeskin tubing molecular weight cut-off 3.5 kDa). Hereafter the peptides were lyophilised.

*SDS-Polyacrylamide gel electrophoresis*

Electrophoresis was performed according to the method described by Laemmli (9). Aliquots of 20 µl of native HHA and UDA, FITC-labelled HHA and UDA as well as various molecular weight markers were placed in 6-8 slots (positions) on 10% and 18% SDS-slab gels (Bio-Rad mini electrophoresis system) and examined by electrophoresis. Electrophoresis of unreduced and reduced samples (2-mercaptoethanol, 5%) was performed at room temperature at a constant voltage of 200 V for approximately 45 min. Hereafter, the gels were stained with 0.1% Coomassie blue R-250 and de-stained in a solution of 40% methanol and 10% acetic acid. The molecular weights of the stained protein bands were determined using molecular weight markers, low-range (2.5 kDa – 45 kDa), high-range (14.3 kDa – 85 kDa). Stability of FITC-labelled peptides before and after passage through vaginal mucosa was verified in our laboratory during a previous study (10). There were no indications that FITC labels dissociated or were enzymatically cleaved from the peptides.

*Permeability Experiments*

Prior to each permeability experiment, vaginal tissue specimens were thawed at room temperature in phosphate buffered saline (PBS, pH 7.4). The diffusion kinetics of FITC-labelled HHA and UDA through thawed frozen vaginal mucosa were then determined. After equilibration of the specimens in PBS, they were carefully cut, so as not to damage the epithelial surfaces, into sections (4 mm in diameter) and then mounted in flow-through diffusion cells (exposed areas 0.039 $cm^2$) with the epithelial surfaces facing upwards. Permeation studies were performed on 7 tissue replicates for each patient, ie

N =35, the latter representing the number of specimens used for statistical analysis. Prior to commencing each permeability experiment, tissue disks were equilibrated for 10 min with PBS (pH 7.4) at 20°C in both the donor and acceptor compartments of the diffusion cells. Following equilibration, the PBS was removed from the donor compartment and replaced with 0.5 ml of 0.5 mM solution of FITC-labelled protein in PBS. PBS at 20°C was pumped through the acceptor chambers at a rate of 1.5 ml/h and collected, by means of a fraction collector, at 2 h intervals for 24 h. The permeability study was performed under sink conditions, i.e. at the completion of each run the concentration of permeant in the acceptor chamber never reached 10% of that in the donor compartment. For the detection of FITC-labelled HHA and UDA, fluorescence was measured (emission: 520 nm and excitation: 497 nm) using a Perkin-Elmer LS50B luminescence spectrometer (Perkin-Elmer, MA, USA).

*De-epithelialisation*

Mucosal surfaces were de-epithelialised by heat stripping to mimic ulceration and the procedure was carried out as follows: a beaker containing distilled water was heated to 80ºC. The thawed vaginal mucosa specimens were then dipped into the beaker for 30 s, using tweezers. Upon removal of the tissue from the water, the uppermost layer was peeled off using another pair of tweezers. These de-epithelialised tissue samples were then cut into 7 pieces, 4 $mm^2$ each, for loading into the donor compartments of the flow-through perfusion apparatus. Previous studies, including histology, have shown heat de-epithelialisation to be more complete and at least as efficaceous as mechanical stripping (10-11).

*Calculation of Flux Values*

Flux (J) values ($pmol.cm^{-2}.min^{-1}$) of the HHAand UDA across the vaginal membranes were calculated by means of the relationship: J = Q/A x t, where Q = quantity of compound crossing membrane (pmoles), A = membrane area exposed ($cm^2$) and t = time of exposure (min).

*Steady-State Kinetics*

Steady state was assumed to have been reached for a particular specimen and chemical compound when no statistically significant differences ($p<0.05$) at the 5% level (t-test with Welch's correction) between flux values were obtained over at least 2 consecutive time intervals.

## RESULTS

The electrophoretograms for HHA, UDA as well as FITC-labelled HHA and UDA are shown in Fig. 1-3. Whereas UDA proved to be a homogeneous preparation of monomer with a molecular weight of ~ 8.5 kDa, HHA was a heterogeneous mixture of protein fractions with molecular weights that ranged between ~ 13 and 52 kDa.

Mean flux values of FITC-labelled HHA and UDA across intact and heat de-epithelialised human vaginal mucosa, versus time are shown in Fig. 4 and 5. Flux values for HHA were between ~3 and 6 $pmol.cm^{-2}.min^{-1}$ during the first 10 h of the experiment. Flux rates for HHA across de-epithelialised vaginal mucosa were ~2x higher than across intact mucosa. Hereafter flux rates decreased and levelled off to levels of ~2-3 $pmol.cm^{-2}.min^{-1}$ between 10 and 24 h, those across de-epithelialised mucosa remaining slightly higher than across intact mucosa. Steady state flux values across de-epithelialised mucosa were reached after ~10 h and for intact mucosa after ~18 h. At commencement of the experiments a total of 250 000 pmol of each lectin was added to the donor chamber of the perfusion apparatus. After 24 h a cumulative amount of ~ 26 pmol of HHA had diffused across intact and ~ 40 pmol across heat de-epithelialised mucosa. This represents ~ 0.011 % and ~ 0.016 % of the initial quantity in the donor cell, respectively.

Fig. 5 shows a steady increase in the mean flux rates for FITC-labelled UDA across de-epithelialised and intact vaginal mucosa. Although the heat-stripped mucosa was only slightly more permeable to UDA during the first 16 h of the experiment, this difference became ~2x higher between 16 and 24 h. The mean flux rate during the first 16 h of the experiment increased to ~ 10 $pmol.cm^{-2}.min^{-1}$ and thereafter to ~ 15 to 25 $pmol.cm^{-2}.min^{-1}$. Steady state flux values across intact and de-epithelialised mucosa were not reached during the course of the experiment. After 24 h a cumulative amount of ~ 114 pmol of UDA had diffused across intact and ~ 157 pmol across heat de-epithelialised mucosa. This represents ~ 0.046 % and ~ 0.063 % of the initial quantity in the donor cell, respectively.

## DISCUSSION

From the electrophoretogram in Fig. 1 it can be seen that native HHA is a heterogeneous mixture composed of tetramers (~52 kDa), trimers (~39

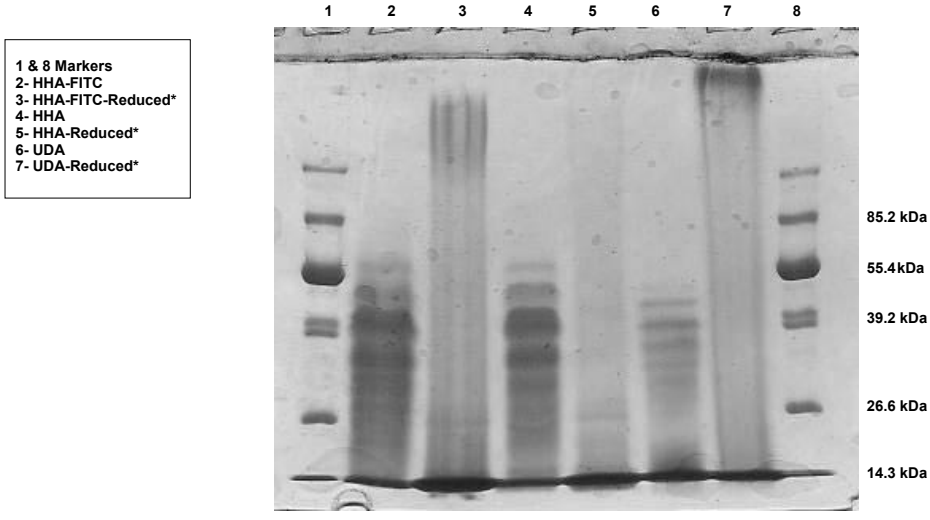


**Fig. 1.** *Electrophoresis of HHA in 10% SDS PAGE.*

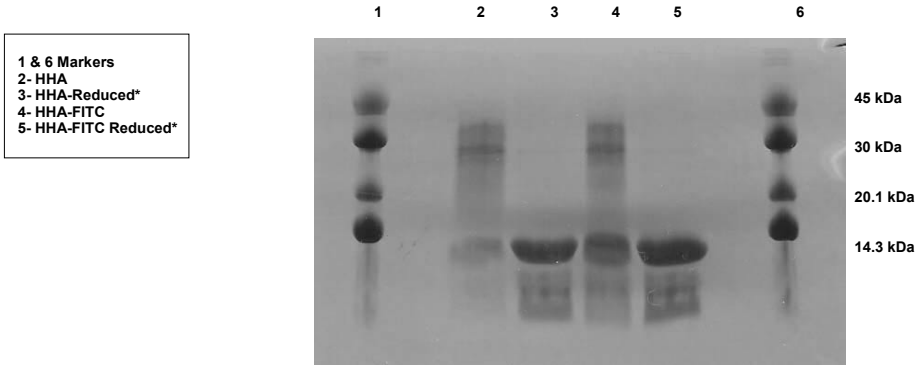


**Fig. 2.** *Electrophoresis of HHA in 18% SDS PAGE with markers of lower molecular weight.*

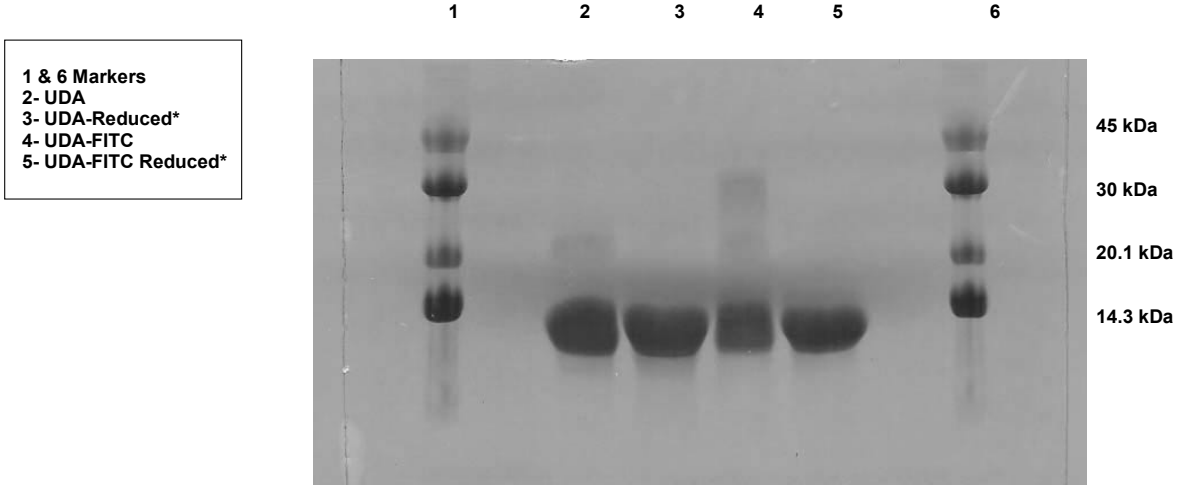


**Fig. 3.** *Electrophoresis of UDA performed on 18% SDS PAGE.*

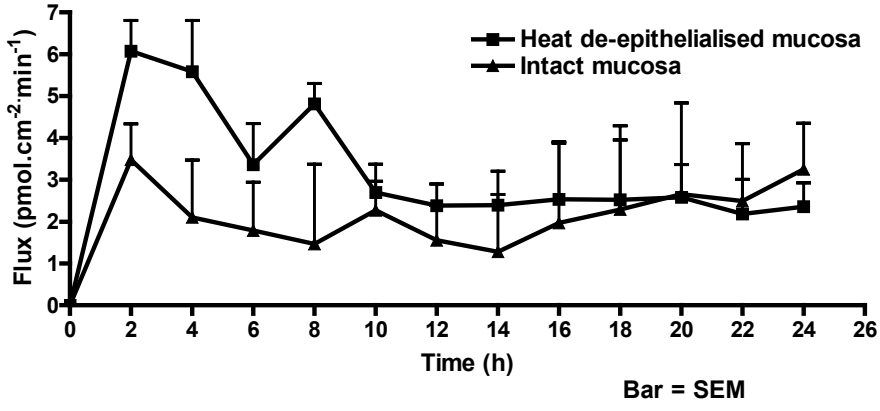


**Fig. 4.** *Mean flux of HHA across heat de-epithelialised and intact human vaginal mucosa.*

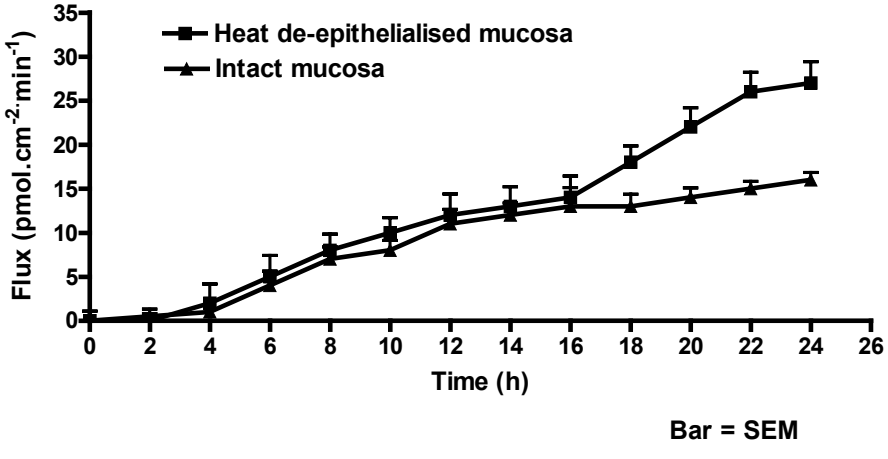


**Fig. 5.** *Mean flux of UDA across heat de-epithelialised and intact human vaginal mucosa.*

kDa), dimers (~26 kDa) and monomers (~13 kDa) (position 4). The subunits in the tetramers, trimers and dimers are not covalently linked as shown in the reduced electrophoretograms. On FITC-labelling the electrophoretogram, remained essentially the same as that for the native product (position 2), although more streaking was observed. On treatment of the HHA and FITC-HHA (positions 3 & 5) with β-mercaptoethanol the internal disulfide bridges (one in each monomeric subunit) were reduced, the majority of the HHA forming small fragments of < 14 kDa (Fig. 1).

By using an 18% SDS-PAGE gel an electrophoretogram of native and FITC-labelled HHA (Fig. 2), similar to that found using the 10% SDS-PAGE gel, was obtained with primarily trimers, dimers and monomers of the lectin being present (positions 2 & 4). Due to the more highly polymerised nature of the gel (18% vs 10%) the HHA tetramers probably remained at the origin and did not enter the gel. Reduction of native and FITC-labelled HHA again resulted in mainly monomers and smaller, molecular mass fragments of < 14 kDa (streaked-out towards the migration front) (positions 3 & 5). From Fig. 1 and 2 it can therefore be concluded that the native HHA material supplied consisted of a mixture of tetramers, trimers, dimers and monomers.

UDA is a small monomeric protein (~ 8.5 kDa). From the electrophoretogram, small quantities of a

relatively high molecular weight band in the region of 20 kDa were observed for native UDA (position 2) (Fig. 3). However, the majority of UDA (>95%) was concentrated in a fairly homogeneous band <14 kDa. This was again the case following reduction of the UDA with β-mercaptoethanol, but the 20 kDa band (positions 3 & 5) was no longer present on the elctrophoretogram. Interestingly enough, FITC-labelling introduced another band in the higher molecular weight region of 30 kDa (position 4). For this finding, we have no plausible explanation, other than that FITC-labelling may produce an anomalous structural change of the protein in the SDS-PAGE gel. Reduction of FITC-labelled UDA again resulted in an almost homogeneous band of <14 kDa (postion 5). The UDA supplied therefore appeared to predominantly consist of monomer (~ 8.5 kDa).

The initial increases in flux rates for HHA across vaginal mucosa may possibly be due to small fragments of the heterogeneous mixture of protein fragments diffusing through the tissue for approximately the first 10 h of the experiments (Fig. 4). Thereafter larger fragments started to diffuse through at a lower flux rate. The ~2-fold higher flux rates after de-epithelialisation of the vaginal mucosa mimics the situation when the integrity of vaginal epithelium is compromised due to disease or local trauma. A similar 2-fold increase in flux rate was also observed for other small peptides in a previous study comparing de-epithelialised and intact mucosa (10). This once more lends support to the hypothesis that the main barrier in vaginal mucosa is situated in the epithelium and that damage to this barrier may have possible implications for increased *in vivo* uptake of microbicides as well as HIV.

The much higher flux rates (~ 5x) for UDA compared to HHA across intact and de-epithelialised vaginal tissue and the slow increase with time, not reaching steady state, is in keeping with the differences in average molecular weight of the two molecules. Similar shapes of flux versus time curves were also found for the diffusion rates of small synthetic peptides in a previous study (10).

In conclusion, both HHA and UDA traverse vaginal mucosa *in vitro*, albeit in small quantities. Considering the relatively large molecular weights of these compounds, this is a significant finding. If infective, traumatic or other damage of the epithelium of the mucosa is present *in vivo*, even higher systemic uptake of these compounds may occur.

## ACKNOWLEDGEMENTS

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DISCLAIMER: Any opinion, findings and conclusions or recommendations expressed in this material are those of the author(s).

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# *IN VITRO* ZIDOVUDINE-INDUCED APOPTOSIS DEMONSTRATED BY A MODEL OF PLACENTAL HISTOCULTURE USEFUL FOR THE STUDY OF THE APOPTOTIC EFFECTS OF ANTIRETROVIRAL DRUGS

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**This study aims to evaluate the use of a placenta histoculture system based on placental villi culture on collagen sponge gels at the liquid/air interface, for the detection of zidovudine-induced apoptosis. Explant cultures from full term placentas were exposed to different concentrations of zidovudine (1, 10, 100 uM) up to 7 days and samples were analysed for apoptosis with different methods after 3, 24, 48 hours and 7 days of exposure. Apoptotic phenomena were demonstrated at 10 and 100 uM zidovudine, more delayed at the lowest concentration: this demonstrates that zidovudine-induced placenta apoptosis *in vitro* is both time- and dose-dependent and that also concentrations near to those achievable *in vivo* might cause placenta apoptosis. This has to be taken into account when considering possible consequences of antiretroviral treatments during pregnancy. Placental histocultures on collagen sponge cells are a reliable tool for the study of antiretroviral-induced toxicity in placenta: in fact, explants cultured for more prolonged periods (up to 7-14 days) in a "physiological milieu" allow the detection of biological effects otherwise not evident in the commonly used, short-term, placental cultures.**

Antiretroviral treatments in pregnant women have been shown to dramatically reduce the risk of HIV vertical transmission: several regimens have been proposed, with Zidovudine being the drug mainly used, alone or in combination with other antiretrovirals (1).

In recent years, also Highly Active Antiretroviral Therapies (HAART) including protease inhibitors (PI) have been introduced in antiretroviral prophylaxis and treatments for HIV- infected pregnant women, as well as the administration of antiretrovirals being increased also during the first three months of pregnancy (2). The possibility that antiretrovirals may cause obstetrical and/or neonatal complications is still a matter of debate (3).

Studies on the possible effects of antiretrovirals on placenta functions and metabolism are therefore important to gain insight into this important field, and *in vitro* methods might be suitable for this purpose.

Zidovudine-induced apoptosis (possibly related to mitochondrial toxicity) in placental villi exposed *in vitro* to this drug has been reported by

*Key words: placenta, apoptosis, histoculture, antiretrovirals, zidovudine*

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Collier et al (4). In that paper, however, apoptosis was demonstrated only at very high Zidovudine concentrations (100 μM) although elevation of reactive oxygen species (ROS) and increased cell death were observed also with lower concentrations (10 uM). The *in vivo* significance of these findings thus remains unknown.

It has to be noted that the limitations of the methods commonly used for culturing placental villi consist in that, generally, viability of the samples is allowed only up to 24-48 hours: Faye et al (5) recently described a method for placental histocultures on collagen sponges capable of maintaining the viability and the complex interaction between the various cell subpopulations at the chorionic villi level for several days. This may, of course, allow the analysis of placental responses to exogenous stimuli for a significantly more prolonged period of time.

With this in mind, in this study we used such a placental histoculture system in order to a) evaluate the adaptability of the method to study the response of placental cells at different concentrations of antiretrovirals, namely zidovudine, and b) test the hypothesis that more prolonged times of placental villi culture exposure to antiretrovirals might allow the detection of placental cell apoptosis also at lower concentrations of drugs.

## MATERIALS AND METHODS

*Placenta samples*

Full term placentas were obtained after elective cesarean section, and were processed as described by Faye et al (5). Briefly, placental chorionic villi were isolated and minced into 2-3 mm blocks, washed extensively with RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FCS), 1% penicillin-streptomycin and 1% L-glutamine (washing medium). All fragments were placed on top of the collagen sponge gels (Condress collagene, Abiogen Pharma SpA, Italy) into wells of 6-well plates with 3 ml/well of RPMI 1640 supplemented with 15% FCS, 1% penicillin-streptomycin, 0.1% gentamycin, 1% amphotericin B, 1% L-glutamine, 1% non-essential amino acids and 1% sodium pyruvate plus different concentrations of AZT (1,10 μM and 100 μM). The fragments were then placed on top of the collagen sponge gels (9 fragments per sponge per well) at the interface between the medium and the air. Histocultures were maintained at 37°C humidified incubator with 5% $CO_2$ for 24 hours. At different time points (1, 3, 24 hours and 7 days of culture) fragments were collected, fixed in 10% formalin, washed in running water, soaked in a graded series of ethanol and embedded in paraffin. Sections were cut (4 mm thick) and prepared for staining.

Hematoxylin–eosin and PAS staining were used for histological diagnosis. Some fragments were stored at -80°C until use.

*Apoptosis detection in placental histocultures*

Apoptosis was analyzed in placenta histocultures by using three different methods: a ) An *in situ* cell death detection kit (In Situ Cell Death Detection Kit, Roche Molecular Biochemicals, Mannheim, Germany) based on the terminal deoxynucleotidyl transferase-mediated dUTP-nickend-labeling (TUNEL) method. The procedures were carried out according to the protocol of the kit. The cells with green staining in nuclei were considered TUNEL positive; b) Immunohistochemical staining for the cleaved caspase-3 (Cell signalling Technology, Inc), that is specific for cells undergoing irreversible apoptosis. Briefly, the sections were mounted on poly-L-lysine-coated slides, deparaffined, incubated in xylene for 20 minutes, followed by washing with decreasing volumes of ethanol, and washed with distilled water and with phosphate buffer saline (PBS) for 10 minutes. The sections were heated in a microwave oven for 15 min at 600W with a citrate buffer (0.01 mol/L, pH6). Endogenous peroxidase was inhibited by exposing sections to 3% $H_2O_2$ for 10 min at RT, and incubated overnight at 4°C with the primary antibody anti-caspases 3 diluted 1:1000. The following day, the sections were washed twice with PBS, incubated with biotinylated IgG for 15 minutes, washed twice with PBS and incubated with streptavidin-peroxidase conjugate (LSAB2 System-HRP, DakoCytomation, Italy). After washing with PBS, the sections were incubated with DAB substrate to stain immunolabelling and then with Mayer's hematoxylin. Sections were covered with mounting medium and analysed with light microscope. Control samples were processed in an identical manner, but the primary antibody was omitted; c) As a third method, western blot analysis for both uncleaved and cleaved caspases 3 was used. The villi fragments were lysed with CHAPS buffer (Cell signalling Technology, Inc) in the presence of protease inhibitors. The lysates were centrifuged at 13,000 rpm for 10 minutes to remove debris. Protein concentration in the placental histocultures were determined with the Bradford protein assay kit (bio-RAD). Protein samples (30 ug) were separated by 15% SDS−PAGE gel and transferred to nitrocellulose membrane, after blocking with non-fat dried milk, the membrane was incubated overnight with specific primary antibody ( anti-caspase 3 diluted 1:1000) to monitor caspase activity. The following day, the membrane was washed and incubated with HRP-conjugated secondary antibody (1:2000) and followed by incubation with lumiglo

substrate. The reactive band was scanned by using the Versadoc detection system (BIO-RAD).

## RESULTS

In this study a novel, recently described, placenta histoculture method was used to investigate the response in terms of apoptosis of placental cells after exposure to different concentrations of zidovudine (1, 10, 100 μM) and at different time points: 3, 24, 48 hours and 7 days (Fig. 1).

Exposure of placental histocultures to low concentrations (1 μM) of zidovudine did not produce changes in the morphology of placental chorionic villi for the whole culture period as demonstrated by hematoxylin/eosin-stain; here, the majority of nuclei were morphologically normal. At the same time, very few positive nuclei were detected by the TUNEL assay in respect to the unexposed control. Western blot analysis and immunohistochemical staining of paraffin-embedded placentas confirmed these results; in fact, a high reactivity for cytoplasmatic procaspase 3 but not for activated caspase 3 was observed.

When placental histocultures were treated with 100 μM AZT a completely different picture was observed; in fact, ematoxylin-eosin stained placental histocultures presented clear evidence of shrinkage and chromatin condensation against the nuclear envelope thus confirming a pyknosis process that is a hallmark of apoptosis; furthermore by using the immunohistochemical method a high positive cytoplasmatic reactivity for active caspase 3 compared to the inactive form was observed. The apoptotic phenomenon was evident in histocultures exposed to 100 μM already after 3 hours of exposure and massive after 24-48 hours of incubation; at the 7$^{th}$ day of culture, virtually all cells appeared apoptotic (Fig. 2).

When placental samples were exposed to 10 μM zidovudine, a low rate of apoptosis, while more extended than in unexposed controls, was observed only after 48 hours of incubation. Nevertheless, at the 7$^{th}$ day of culture massive apoptotic phenomena were demonstrated also at the 10 μM concentration (Fig. 3).

## DISCUSSION

The present study aims to evaluate the use of

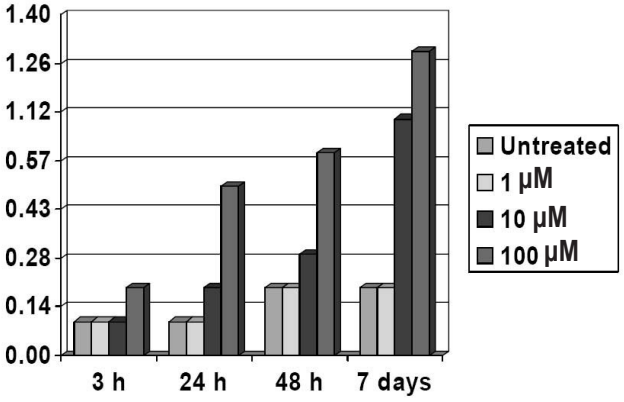
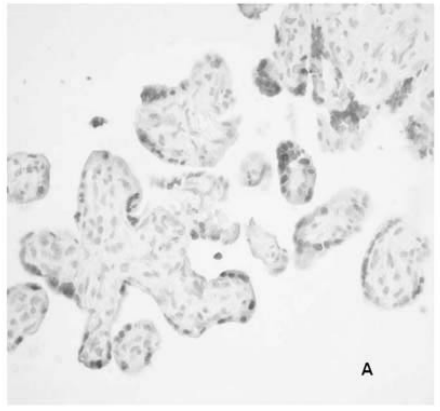



**Fig. l.** *Cleaved caspase 3 expression in placental histocultures in response to treatment with different concentrations of zidovudine.*



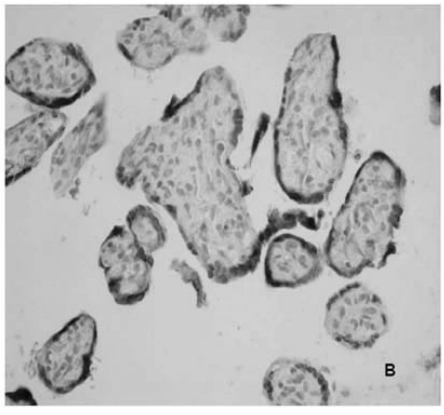




**Fig. 2.** *Immunohistochemistry for cleaved caspase 3 in placental histocultures treated with100 μM of zidovudine for 24 hours (A) and 7 days (B) (original magnification X40). Images are visualized with immunoperoxidase staining using cleaved caspase-3 antibody with a diaminobenzidine developer (dark colour) and a haemotoxylin immunostaining.*

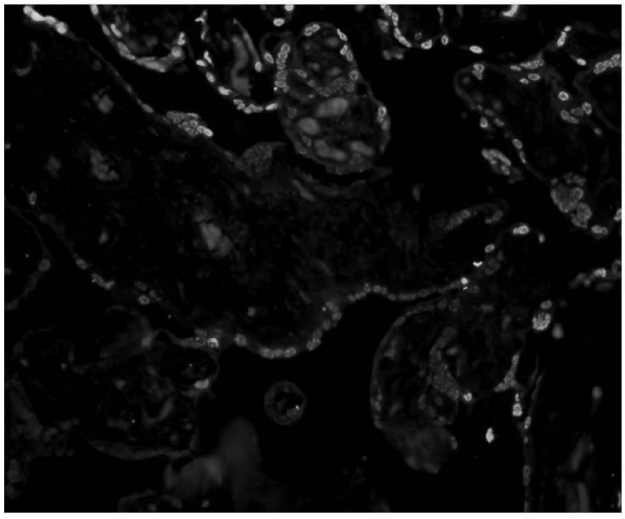


**Fig. 3.** *Immunofluorescence for apoptotic nuclei in zidovudine-treated placental histocultures by using the modified terminal deoxynucleotidyl transferase-mediated dUTP nick and labelling (TUNEL).*

a novel method of placental histoculture for the evaluation of antiretroviral-induced apoptosis.

Placental villi were exposed in the experiments to three different zidovudine concentrations; 1, 10 and 100 μM. While no changes could be observed after exposure to 1 μM zidovudine, apoptosis was clearly demonstrated in samples exposed to 10 and 100 μM zidovudine. The apoptotic phenomena were greater and more precocious at the highest concentration: these findings clearly indicate that apoptosis occurs *in vitro* in placenta samples exposed to zidovudine and that this event is correlated both to the dose and to the time of exposure to the drug.

Our observations confirm and extend those from Collier et al (4), that demonstrated *in vitro* zidovudine-induced apoptosis in placenta exposed to high concentrations of the drug. In this study, however, also lower zidovudine concentrations (10 μM) were shown to induce apoptosis, although after a more prolonged time of exposure. We believe that these discrepancies may be related to the type of placenta sample culture used in our study (on collagen sponge gels), allowing a prolonged period (7 days) of culture of the villi. In the experiments performed by Collier et al, instead, the cultures had to be stopped after only 24 hours: this might have caused a hindrance to the detection of apoptosis at low zidovudine concentration that, in fact, appeared later in our experiments.

This finding seems to be relevant: zidovudine clinical steady state concentrations are 0.5-5 μM but large intra-individual variations have been demonstrated up to 30 μM (6). In addition, umbilical cord blood levels in pregnant women treated with zidovudine are around 120% of their corresponding peripheral blood concentrations (7): thus, placenta is conceivably exposed *in vitro* to zidovudine concentrations similar to those reported to cause apoptosis in our study (10 μM). It is therefore conceivable that the apoptotic phenomena also occur *in vivo*, and their relevance in the outcome of pregnancy in HIV-infected women should be addressed.

Accumulating evidence indicates that increased placental apoptotic phenomena are associated with high risk of both preeclampsia and intrauterine growth retardation (8-11), and these pregnancy complications are also suggested to be increased in HIV infected pregnant women treated with antiretrovirals (12-13). Further studies are therefore needed to investigate the effects of different antiretroviral drugs (either alone or in combination), on placental cells.

The placenta histoculture method used in this study was suitable and reliable for the purpose of the study and could be therefore proposed for studies on the consequences of placenta exposure to antiretroviral drugs; the possibility, in fact, to achieve a physiological milieu (with intact cell interactions) for such a prolonged period of time may be very useful to detect and characterize drug-related time-dependent effects on exposed placental cells.

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# NEBIVOLOL INCREASES ATRIAL NATRIURETIC PEPTIDE/SYSTOLIC BLOOD PRESSURE RATIO IN HYPERTENSIVE PATIENTS

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**The aim of this study is to evaluate the medium term treatment effects of the beta-blocker nebivolol on the endogenous anti-hypertensive and anti-inflammatory agent Atrial Natriuretic Peptide (ANP) plasma levels in patients with moderate uncomplicated essential hypertension. The drug was given orally for 30 days to 25 patients. The daily dose was 5 mg once every morning. Quantitative determination of ANP was made by Radioimmunoassay (RIA). At the end of this clinical trial, plasma ANP levels showed a statistically non significant tendency to increase by 10.99% while both systolic (SBP) and diastolic (DBP) blood pressure as well as the diameters of the left cardiac cavities showed a statistically significant decrease. What was interesting was the fact that there was a statistically significant increase of 44.7% in the ratio ANP/SBP following the reduction of arterial blood pressure. This increase was the most important and essential finding as it indicated a real increase of ANP plasma levels. This result was obtained even though pressure and afterload, the homologous mechanical stimulatory parameters for ANP secretion, of the left cavities were diminished. These findings provide strong indications which were supported by several previous trials, that the increase in plasma ANP following the administration of beta-adrenergic blockers to hypertensive patients was a primary effect of beta blockade and not a mechanical one secondary to a negative inotropic action on the left ventricle. This primary action contributed to the anti-hypertensive effect of nebivolol during this trial.**

It is known that the short-term effect of antihypertensive drugs is due to their action on cardiac output and/or peripheral resistance. However, their long-term effect is due to changes in the pressure/natriuresis relationship. No conventional hemodynamic effect can provide a satisfactory explanation for the chronic long-term antihypertensive efficacy of β-adrenergic blockers. Thus, it is quite reasonable to try to explain the effects of the above drugs on the arterial pressure by evaluating their action on factors that control pressure-natriuresis. Such a factor is Atrial Natriuretic Peptide (ANP) which also possesses anti-inflammatory properties (1). Furthermore, the links between inflammation and cardiovascular diseases, especially hypertension, are well documented (2).

*Key words: nebivolol, atrial natriuretic peptide, hypertension, β-adrenergic blockers, ANP/SBP ratio*

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The role of this factor on the anti-hypertensive action of many classes of β-adrenergic blockers has been evaluated and proved /confirmed in clinical trials during the last fifteen year period by the above-mentioned Department of the Medical School of the Aristotle University of Thessaloniki (3-6).

As new β-adrenergic blockers are being introduced, it is reasonable to investigate a possible role of ANP upon the anti-hypertensive action of such agents which are presented with combined actions. Nebivolol is a highly selective blocker of $\beta_1$-adrenergic receptors and, in addition, it stimulates the release of nitric oxide (NO) from the endothelium (7).

Thus, this study aims to investigate the medium-term anti-hypertensive effect of nebivolol in relation to plasma ANP concentration changes in patients with moderate degree uncomplicated essential hypertension.

## MATERIALS AND METHODS

*Study population*

In this study 25 patients (12 males and 13 females, mean age 54.7±12.2 years, range 33-74) with mild to moderate uncomplicated essential hypertension were enrolled. All patients satisfied the inclusion criteria for treatment with the $\beta_1$ adrenergic blocker nebivolol. They had resting heart rate of ≥70/min with normal atrioventricular conduction. Their renal function was normal, as detected by conventional biochemical tests, or by radioisotopic studies whenever it was necessary. In all patients, left ventricular and atrial dimensions as well as systolic left ventricular function as detected by the echocardiogram, were normal. There was no evidence of ischemia on the surface resting ECG. Also, whenever it was necessary, ischemia was ruled out using myocardial perfusion imaging exercise (SPECT, Thallium-201).

Some of the patients were newly diagnosed as having hypertension and were untreated; in the rest, all anti-hypertensive drugs were safely discontinued two weeks before enrollment in the trial, since their hypertension was mild to moderate. The trial was performed according to ethics requirements. Patients were properly informed regarding the objectives of the trial and each signed a form of consent. After a washout period of 15 days for those who were previously on anti-hypertensive treatment as well as the rest who had been on observation and behavioral modification, if blood pressure remained higher than 150/95, nebivolol administration was started. Just prior to nebivolol treatment, an echocardiogram was performed on all patients and the dimensions of the left ventricle and the left atrium were measured on the M-mode following the conventional methodology in the left parasternal location using the long axis view. The values shown in the tables are mean values of three to four measurements. P wave duration was also measured using the signal average electrocardiogram (SAECG) with the patient being in the supine position.

The duration of the atrial electrogram expressed by the filtered signal in the SAECG indicates the time from the initiation till the end of depolarization of the atrial myocardium. At the end of the measurements, the patients were kept in a supine position for 30 minutes. A venipuncture was performed from a peripheral vein to determine the concentration of plasma ANP. Blood pressure was measured using a mercury sphygmomanometer in the seating position from the right arm.

The patients received nebivolol from the company Menarini (tablets of 5 mg once every morning). On the 30$^{th}$ day of treatment, the same parameters mentioned above were measured in the same sequence three hours after taking the morning dose. ANP was measured again in blood specimens taken by venipuncture three hours after administration of the last drug dose.

Control groups running in parallel and consisting of patients receiving an anti-hypertensive drug of another category were not included in this study because drugs such as β-adrenergic blockers, ACE inhibitors and calcium entry blockers have been reported by some of the authors and other researchers to increase ANP plasma levels (3-6, 8-15).

Mean Pressure (mP) is derived from the equation mP= Diastolic Pressure+1/3 Differential Pressure. Resistance (R) is approximately derived from the equation R= mP-mPxRA/CO in Wood´s units. MPxRA is the mean right atrial pressure considered approximately 10 mmHg while CO represents the cardiac output (16).

*ANP measurement*

Blood samples for plasma ANP level determination were collected in pre-chilled tubes containing EDTA-2Na (1.5 mg/ml blood) and aprotinin (500KIU/ml blood), placed on ice, and immediately centrifuged at 1500 g for 10 min. at 4$^{o}$C. Plasma was frozen and stored at –70$^{o}$C until the assay was performed. Quantitative determination of human ANP was made by radioimmunoassay procedure (RIA) combined with an extraction step, using reagents supplied by Nichols Institute Diagnostics B.V (California USA). Determination by RIA was performed by using an ANP ($^{125}$J) radioimmunoassay (RIA) system according to the manufacturer's instructions. Intrassay and interassay variations were 3.9% and 9.7% respectively. The results obtained (mean ± SD) were expressed as pg/mL. Normal values calculated in 10 healthy male individuals, mean age 51 years (range 30-60), in our laboratory were 35.3 ± 9.5 pg/mL (17-19).

*Statistical analysis*

The values obtained for each parameter, before and after treatment, were expressed in the form of mean ±1 standard deviation and the statistical evaluation was performed using the Students paired t-test as well as the Wilcoxon test because the distribution was not always regular.

## RESULTS

Mean plasma ANP levels rose after treatment by

**Table I.** *Changes in the parameters studied before and after 30 days of treatment with nebivolol (means ± SD, n: 25).*

| Parameters | Before Treatment | After Treatment | Units | % changes | Statistical Significance |
|---|---|---|---|---|---|
| SBP | 167.24±7.57 | 126.20±10.33 | mmHg | -24.5 | p< 0.001 |
| DBP | 104.00±8.77 | 83.40 ±8.12 | mm/Hg | -19.8 | p<0.001 |
| SV | 111.10±15.5 | 98.10±12.75 | ml | -11.70 | p<0.05 |
| HR | 75±3.2 | 64±2.5 | pulse/min. | -14.66 | P<0.05 |
| CO | 8.333±1.17 | 6.278±0.69 | L /min | -24.66 | P<0.05 |
| R | 13.82±1.70 | 13.96±1.76 | Wood's units | -5.4 | p>0.1 |
| mP | 125.08±7.50 | 97.67±8.80 | mmHg | -21.91 | P<0.05 |
| LVESD | 3.48±0.68 | 3.39±0.68 | cm | -2.58 | p~0.001 |
| LVEDD | 4.96±0.55 | 4.82±0.55 | cm | -2.82 | p<0.001 |
| LVEF | 60.99±8.15 | 62.25 ±8.10 | % | +2.06 | p~0.05 |
| P-Waves | 136.32±31.8 | 32.52 ±27.84 | Msec | -2.78 | p>0.1 WT=p<0.01 |
| LAD | 3.778±0.36 | 3.680±0.33 | cm | -2.59 | p<0.05 |
| ANP | 42.74±17.28 | 47.44±21.75 | pg/mL | + 10.99 | p>0.1 WT=p>0.05 |
| ANP/SBP | 0.257±0.10 | 0.372 ±0.16 | % | +44.7 | p~0.01 WT=p<0.01 |

*SBP=systolic blood pressure; DBP=diastolic blood pressure, ANP=atrial natriuretic peptide (plasma concentration); LVESD=left ventricular end systolic diameter; LVEDD=left ventricular end diastolic diameter; LVEF=left ventricular ejection fracture; LAD= left atrial diameter; SV=Stroke Volume; HR=Heart Rate; CO=Cardiac Output; R=Resistance; mP=mean arterial Pressure; WT= Wilcoxon test*

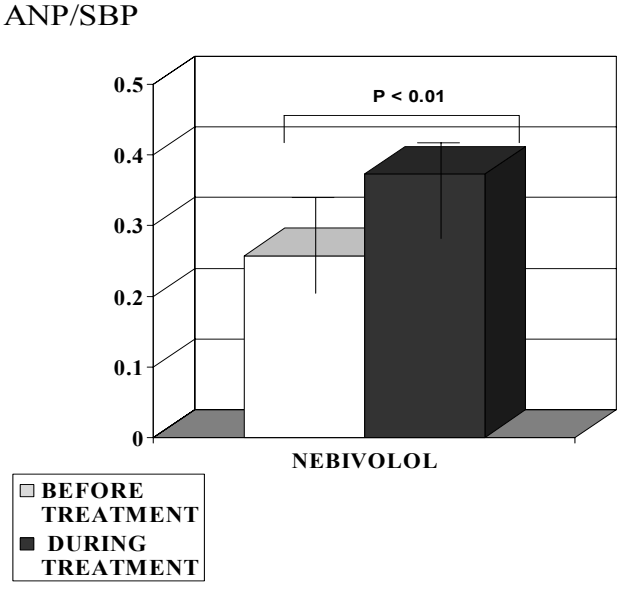


**Fig. 1.** *Changes of ANP/SBP ratio on treatment with nebivolol. ANP (Atrial Natriuretic Peptide), SBP (Systolic Blood Pressure).*

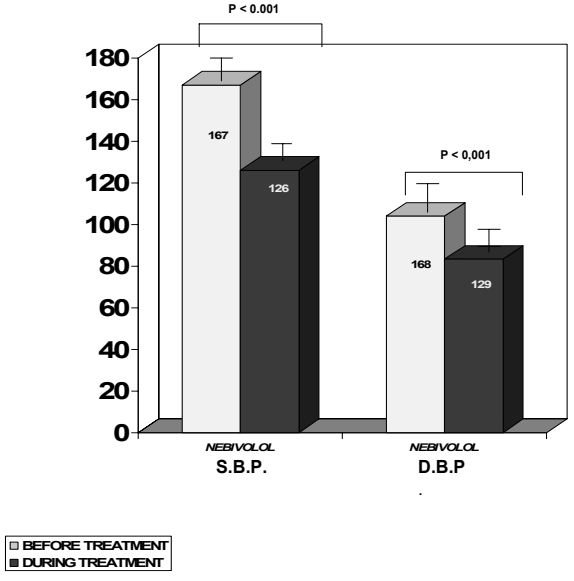


**Fig. 2.** *Blood pressure changes on treatment with nebivolol.*

10.99% (from 42.74±17.28 pg/ml to 47.44±21.75 pg /ml) but this increase was not statistically significant. Both systolic blood pressure (SBP) and diastolic blood pressure (DBP) were decreased by 24.5% (from 167.24±7.57 mmHg to 126.20±10.33 mmHg) and by 19.8% (from 104.00±8.77 mmHg to 83.40±8.12 mmHg) respectively and in a statistically significant way. Left cardiac cavitiy diameters were also decreased in a statistically significant way. Thus, left atrial diameter was decreased by 2.59% (from 3.77±0.36 cm to 3.68±0.33 cm), left ventricular end systolic diameter (LVESD) was decreased by 2.58% (from 3.48±0.68 cm to 3.39±0.68 cm) and left ventricular end diastolic diameter (LVEDD) was decreased by 2.82% (from 4.96±0.55 cm to 4.82±0.55 cm). The ratio ANP/SBP was also increased by 44.7% (from 0.257±0.10 to 0.372±0.16) in a statistically significant way, and that was the more important and essential finding. Left ventricular ejection fraction (LVEF) increased minimally by 2.06% (from 60.99±8.15 to 62.25±8.10) but within the limits of statistical significance All the results are shown in detail in Table I and Fig. 1 and 2.

## DISCUSSION

As expected, nebivolol treatment resulted in a remarkable reduction of both systolic and diastolic pressures by 24.5% and 19.8% respectively (Fig. 1). Left ventricular and atrial diameters decreased marginally but with statistical significance due to the concordant variations, whereas LVEF increased minimally within the limits of statistical significance (Table I). The above findings exclude any final negative inotropic effect of nebivolol.

During treatment with nebivolol no statistically significant change of P wave duration on atrial electrogram was observed as it was detected by the SAECG. P wave duration provides indirect information concerning the length of the cardiac fibers. This information is derived from the changes in the time that is needed for the termination of the atrial electrical depolarization. The main factors that regulate the time are the velocity of intra-atrial conduction and the length of the pathway. Since β-adrenergic stimulation exerts positive dromotropic effect on the myocardium, its inhibition would be expected to prolong the atrial activation time. Absence of such changes in the ECG suggests that atrial activation time remained unaltered and consequently that the length of the pathway might be shortened, underlying reduction in the dimensions of both atria. Similar findings in the echocardiogram, where there was a marginal but statistically significant decrease in the size of left atrium, further indicate a reduced length of the pathway.

The plasma ANP level mean values were slightly higher than our laboratory's normal values before treatment and showed a statistically non-significant tendency to increase by 10.99% during treatment with nebivolol. Comparing the increase in the ANP levels to the SBP gives rise to the main finding of this trial which is a statistically significant increase by 44.7% in the ratio of plasma ANP to SBP following the reduction of arterial blood pressure (Table I, Fig. 1 and 2). Since systolic blood pressure is positively related to the mechanical stimulus affecting ANP secretion, it is reasonable that a reduction of plasma ANP concentration should be expected. The diverting behavior of ANP and blood pressure as indicated by the variation of ANP/SBP ratio along with the decrease of the left cardiac cavity dimensions strongly support the concept of a primary stimulation for ANP secretion by the action of nebivolol.

This is a very important finding because the relation between the plasma levels of ANP and the systolic blood pressure rate reflects mechanisms of the anti-hypertensive action of the drug after a medium term treatment. Nebivolol follows the same rule as β-adrenergic blockers of older generation as well as anti-hypertensive drugs of other classes (3-6, 8-15).

In conclusion, these findings clearly suggest that the increase in plasma ANP after the administration of β-adrenergic blockers to hypertensive patients is a primary effect of beta blockade and not a mechanical one secondary to a negative inotropic action on the left ventricles and the atria.

An additional mechanism for an increase of ANP plasma levels could be its reduced catabolism. The latter possibility might be suggested by the observation that spontaneously stroke-prone rats that were treated with β-blocker showed increased plasma levels of ANP and decreased mRNA levels of Natriuretic Peptide–C receptor which is responsible for the clearance of ANP (20).

Irrespective of the mechanisms involved, the principal message from this study is that nebivolol, classified as a new generation β-adrenergic blocker with polymorphic actions, exerts part of its anti-hypertensive action by increasing ANP levels.

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# EXPERIMENTAL STUDY OF THE EFFECTS OF THE HYPOTHALAMIC-PITUITARY-THYROID AXIS ON PLATELET FUNCTIONAL ACTIVITY

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**The hormones of the hypothalamic-pituitary-thyroid axis exert various effects in organism, but their influence on the functional activity of platelets is relatively unknown. To establish the effect of the hormones of the hypothalamic-pituitary-thyroid axis on the functional activity of platelets by means of determining the plasma level of beta-thromboglobulin (β-TG) and platelet factor 4 (PF4) a study was conducted on 40 white male rats of the "Wistar" breed. The necessary blood volume was obtained under ether narcosis by cardinal puncture in CTAD-test-tubes. Using Diagnostica Stago (France) tests, by means of enzyme-immune test, the level of two of the most informative platelet secretion markers was determined. The hormones: Thyreotropin releasing hormone (0.06 mg/kg bw), Thyroid stimulating hormone (1 MU/kg bw), Trijodthyroninum (0.08 mg/kg bw), Thyroxin (0.08 mg/kg bw), applied s.c. on three consecutive days strongly reduce the plasma level of β-TG ($p<0.001$) and PF 4 ($p < 0.001$). The reduced level of β-TG and PF 4 in the plasma is an indicator of their reduced functional activity, which is one of the determinants for the development of hemocoagulation. The functional activity of platelets in rats, determined by the level of β-TG and PF 4, is significantly suppressed by all hormones of the hypothalamic-pituitary-thyroid axis.**

In experimental and clinical practice, the most common markers for estimation of the functional activity of platelets are the plasma levels of beta-thromboglobulin (β-TG) and platelet factor 4 (PF 4). The normal β-TG:PF4 ratio in the plasma is approximately 5:1 (1). The increased functional activity of platelets has a triggering significance for platelet-formation, which is a significant problem in contemporary medical practice and theory. There are many diseases, in which the level of the functional activity markers mentioned increases: atherosclerosis (1-3), hypertonic disease (4-7), atrial fibrilation (8-10), liver cirrhosis (11-13), diabetes mellitus (14-15), depression (16-17), etc. Increased levels are also found during pregnancy (18-21). At the same time, data on the effect of the hormones on platelet secretion reaction are scarce, and as far as the effects of the hormones of the hypothalamic-pituitary-thyroid axis are concerned, there are no data. On these bases the objective of our research was formulated: to study the effects of the Thyrotropin releasing hormone (TRH), Thyroid stimulating hormone (TSH), Liothyroninum (Triiodothyroninum – $T_3$) and Levothyroxinum (Thyroxin – $T_4$) on the functional activity of platelets in rats by determining the plasma level of β-TG and PF 4.

*Key words: beta-thromboglobulin, hypothalamic-pituitary-thyroid axis, platelets and platelet factor 4*

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## MATERIALS AND METHODS

*Animals and hormone injection*

We carried out the research on 40 white male Wistar rats, weighing 200–220 g, following the requirements of the European Convention for the Protection of Experimental Animals (Protection of animals used for experimental purposes, Council Directive 86/609/EEC of November 1986). The feeding was carried with standard briquette food and water ad libitum. The animals were divided into five identical groups – one control group and four experimental groups, eight animals in each. The hormones were injected s.c. once daily for three consecutive days. The first group of rats was injected with TRH, acetate salt (Sigma, minimum 97%), in a dose of 0.06 mg/kg bw; the second group by bovine TSH, (in a substance with activity of 2 MU/mg, Sigma), in a dose of 1 MU/kg bw, the third and the forth group – respectively by $T_3$ and $T_4$ (substances produced by VEB Berlin – Chemie, Germany), in a dosage of 0.08 mg/kg bw. The doses used were determined, on the bases of data from bibliographical sources, and through preliminary analyses for determination of the logarithmic dose–effect dependence. The control group animals were injected by physiological solution (a solvent of the hormones) according to the same schedule and volume per kg bw.

*Drawing blood and obtaining blood plasma*

After monitored aether narcosis, by a quick cardinal puncture, 4.5 ml of blood was drawn, and then placed in a CTAD tune (a test-tube containing multi-component anticoagulant mixture: citrate, theohylline, adenosine, dipyridamole). We placed the test-tubes in a freezing bath for 15 minutes, after which they were centrifuged for 30 minutes at 3000 revolutions/min in a freeze centrifuge (at temperatures from +2ºC to +8ºC). We aspirated about 1/3 of the middle part of the supernatant. This is the so-called "CTAD" plasma, which is poor in platelets.

*Determining β-TG and PF 4*

The studied indices β-TG and PF 4 were determined by Diagnostica Stago (France) test through enzyme-immune tests. Each index was determined twice in 200 µl volume of CTAD plasma and the average value was recorded. Plasma with data of hemolysis was not used.

*Statistical analysis*

All data obtained were analyzed by variation analysis, using the Student-Fisher t-test .

## RESULTS

*Influence of TRH, TSH, $T_3$ and $T_4$ on the level of β-TG*

The results are presented in Fig. 1. TRH reduces the level of β-TG by 38.43 % ($p < 0.001$), TSH – by 55.57 % ($p < 0.001$), $T_3$ – by 30.95 % ($p < 0.001$) and $T_4$ – by 57.09 % ($p < 0.001$).

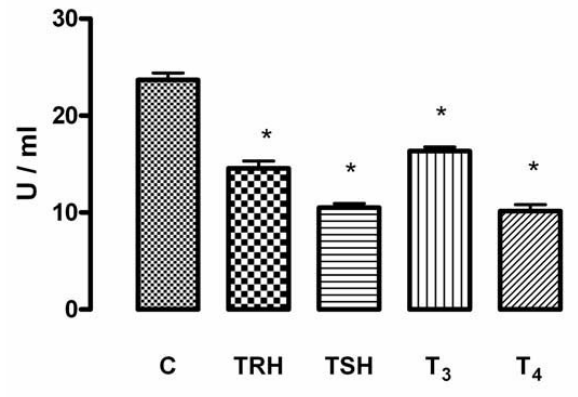


**Fig. 1.** *Effects of TRH (0.06 mg/kg b.w.), TSH (1 MU/kg b.w.), $T_3$ (0.08 mg/kg b.w.) and $T_4$ (0.08 mg/kg b.w.) applied s.c. to white male Wistar rats once daily on three consecutive days on the level of β-TG. U/ml – plasma level of β-TG TRH – thyrotropin releasing hormone, TSH – thyroid stimulating hormone, $T_3$ – triiodothyroninum, $T_4$ – thyroxin, C – control group rats, injected with physiological solution * - $p < 0.001$*

*SH, $T_3$ and $T_4$ on PF 4*

The results are presented in Fig. 2. TRH reduces the level of PF 4 by 43.85% ($p<0.001$), TSH – by 67.52 % ($p<0.001$), $T_3$ – by 45.86 % ($p<0.001$), and $T_4$ - by 57.68 % ($p<0.001$).

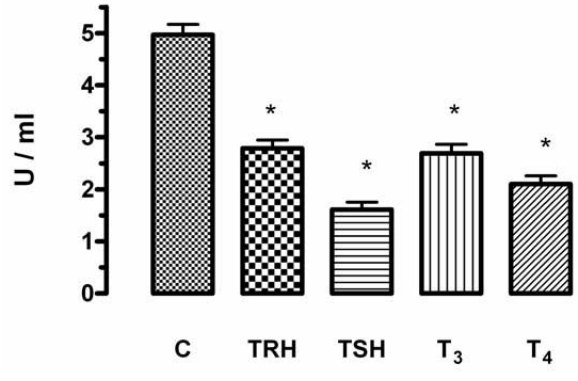


**Fig. 2.** *Effects of TRH (0.06 mg/kg b.w.), TSH (1 MU/kg b.w.), $T_3$ (0.08 mg/kg b.w.) and $T_4$ (0.08 mg/kg b.w.) applied s.c. to white male Wistar rats once daily on three consecutive days on PF 4. U/ml – plasma level of PT TRH – thyrotropin releasing hormone, TSH – thyroid stimulating hormone, $T_3$ – triiodothyronine, $T_4$ – thyroxin, C – control group rats, injected with physiological solution * - $p < 0.001$*

## DISCUSSION

It is clear from Fig. 1 that the level of β-TG in the plasma is strongly reduced under the influence of TRH, TSH, $T_3$ and $T_4$ in comparison to the control group of rats. Having in mind that β-TG is a marker of platelet secretion reaction (22-26), and thus of their functional activity, it follows that this activity is suppressed. Therefore, we can assume the hormones of the hypothalamic-pituitary-thyroid axis are significant regulators of platelet functional activity. Together with the data exposed so far, it is also notable that TRH and $T_3$ have similar effects in terms of the secretion of β-TG, while the effects of TSH and $T_4$, though unidirectional with the latter, are significantly better expressed. By analogy with the data exposed above, it could be said that the suppressed functional activity of platelets displays variations, identical to those of the reaction of their secretion.

The analysis of the data on the results of the effects of TRH, TSH, $T_3$ and $T_4$ shown in Fig. 2, gives evidence that the level of PF 4 in the plasma of the experimental animals is severely reduced. There is a significantly reduced secretion of this marker as well, while the functional activity of the platelets is suppressed. TSH and $T_4$ cause a reduction in the secretion of PF 4 by the platelets to a greater extent, hence their functional activity, while TRH and $T_3$ suppress it, albeit to a smaller extent.

The results of the suppressed secretion of β-TG and PF 4, the main markers of both the reaction of secretion and the functional activity of platelets, correspond to the unpublished data that we have obtained, according to which the hormones considered cause an expressed hypocoagulability. The intimate mechanism of these phenomena can only be commented hypothetically, bearing in mind the scarce and contradictory data published, mostly based on clinical observations. In this respect, some of the most probable mechanisms could be speculatively suggested. It is well-known that some statins may reduced β-TG and PF 4 plasma concentrations (27-29), while others decrease the platelet functional activity (30). In conclusion, the functional activity of the platelets in rats, determined by the levels of β-TG and PF 4, is suppressed to a significant extent by TRH, TSH, $T_3$ and $T_4$. Bearing in mind, on one hand, that platelet functional activity has a triggering effect on the coagulation process, and, on the other hand, the wide application of these hormones in clinical practice, it could be stated that the results obtained establish the necessity for detailed investigation of the effects of the hypothalamic-pituitary-thyroid axis on hemostasis.

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# CLINICAL AND LABORATORY CHANGES IN THE UNCOMPLICATED COURSE OF ARTHROSCOPIC ANTERIOR CRUCIATE LIGAMENT (ACL) RECONSTRUCTION: A PROSPECTIVE OBSERVATIONAL STUDY IN 58 PATIENTS

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**Knowledge of post-operative clinical and laboratory changes in the uncomplicated course of ACL reconstruction could assist orthopaedic surgeons in making a timely diagnosis of septic arthritis. A total of 58 candidates to ACL reconstruction were enrolled in this study according to inclusion/exclusion criteria. Pre- and post-operative changes on 1st, 3rd, 7th, 15th, and 30th day were collected for skin body temperature (SBT), knee pain (KP), white blood cell (WBC) count, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP). Post-operative joint effusion was recorded when inhibiting quadriceps contraction. The post-operative changes were compared with baseline values at each time point in the whole sample and in the subgroups of patients receiving bone- patellar-tendon-bone (BPTP) or hamstring tendon (HT) autograft. Follow-up at six month was considered adequate to rule out surgery-related septic arthritis. Mean SBT significantly increased on 1st, 3rd and 7th post-operative day and returned to values not differing from baseline on 15th and 30th day. Median KP was significantly elevated during the first month. Joint effusion was observed on 7th post-operative day in 52% of patients. Mean WBC count significantly increased on 1st, 7th and 15th day while no difference from baseline was observed on 3rd and 30th day. Mean ESR was significantly elevated on 1st day, increased on 3rd day and peaked on 7th day. ESR decreased on 15th and 30th day but remained significantly higher than baseline. Mean CRP significantly increased on 1st day, peaked on 3rd day and decreased on 7th day, while levels on 15th and 30th day did not differ from baseline. The SBT, KP, WBC count, ESR and CRP changes stratified in relation to the type of autograft showed the same variation trend as in the whole sample. Close clinical surveillance may be advisable when SBT, occurrence of joint effusion and CRP levels deviate from the described reference trend two weeks after surgery.**

Although the natural history of ACL-insufficient knees is unclear (1-2), it is generally agreed that recurrent episodes of symptomatic instability would lead to an accrual of joint damage (1, 3), particularly if a meniscectomy has been performed following ACL tear (4). The indication for ACL reconstruction includes the desire of recreational or professional athletes to return promptly to the pre-injury activity level or the persistence of symptomatic instability following rehabilitation and activity level modifications (1, 3).

The onset of septic arthritis, the reported rate of

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which following arthroscopic ACL reconstruction ranges from a minimum of 0.14% (5) to a maximum of 1.7% (6), may be devastating owing to the risk of graft failure and joint damage.

The diagnosis of joint infection after ACL surgery is initially based on a high suspicion index. A definite diagnosis is made on average within 3 weeks from surgery (5-9) and a variable amount of diagnostic delay has been reported (5, 7). Clinical signs and laboratory tests that can substantiate clinical suspicion include joint effusion, pain at wound and with knee motion, erythema, drainage and fever, elevated white blood cell (WBC) count, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) (6-8). However, a definite diagnosis can be made when pathogens are found in cultured aspirates and/or in intra-operative tissue cultures (5-9). Microorganisms reported to be responsible for ACL surgery-related infections include both gram-positive and gram-negative bacteria (6-9). The former are most commonly represented by *Staphylococcus aureus* and coagulase-negative staphylococci such as *Staphylococcus epidermidis* as well as by *Propionibacterium acnes* and *Corynebacterium*. The latter include *Pseudomonas aeruginosa* and *Enterobacteriaceae*.

The diagnostic challenges are compounded by two factors. Firstly, hemarthrosis, which is a frequently early and relatively mild complication after ACL reconstruction (10), can activate a cascade of acute-phase reactants ultimately leading to local and systemic inflammatory signs. Secondly, surgery-related tissue damage itself primes an acute-phase response. Thus neither joint pain, swelling, effusion, limited function and fever nor laboratory test changes are exclusive to joint infection.

Scant information regarding the uncomplicated course of ACL surgery exists in the literature. One paper focused on post-operative ESR and CPR changes in infection-free patients undergoing ACL reconstruction (11). Another study compared ESR and CPR levels in ACL-reconstructed patients developing infection with a small number of non-infected controls (7).

The present study investigated the early post-operative changes of clinical signs and laboratory tests in patients undergoing ACL reconstruction who did not develop infection of the knee joint. The purpose is to supply reference values with which clinical signs and laboratory tests could be compared when septic arthritis is suspected.

## MATERIALS AND METHODS

A prospective observational study was conducted on ACL-insufficient candidates to arthroscopic reconstruction. Inclusion criteria were unilateral chronic ACL deficiency with or without associated meniscal and/or focal chondral lesions and a normal function of the opposite lower limb. The ACL tear was classified as chronic 6 weeks after initial knee injury (12). Exclusion criteria were acute ACL tear, severe (Outerbridge grade III or IV) and diffuse chondral lesions, concomitant knee major (grade III) ligamentous injuries and/or fractures requiring surgery, previous ACL reconstructive surgery and infectious, neoplastic, rheumatic and hematological disorders. Patients showing abnormal ESR and CRP values were also excluded even when no systemic disease was found after diagnostic work up.

The indication for arthroscopic reconstruction was symptomatic knee instability after a rehabilitation program in patients unwilling to change their activity level or despite activity modification. The pre-operative diagnosis of ACL insufficiency was based on physical examination (positive Lachman and pivot-shift tests) and testing of tibial anterior translation (TAT) measured by KT-1000 knee ligament arthrometer (MEDmetric, San Diego, California, USA) at 67 N, 89 N and at maximal manual traction (MMT). Testing was suggestive of ACL tear if TAT was > 10 mm or if the side-to side difference (SSD) was > 3 mm at MMT (13). Knee function was assessed by the International Knee Documentation Committee (IKDC) Examination Form (14). Diagnostic arthroscopy preceded autograft harvesting in all patients on the day of surgery.

All patients received antibiotic prophylaxis (2 g cephazolin by intravenous route 30 minutes before surgery and 1 g repeated 3 times a day on the first post-operative day). Anti-thromboembolic prophylaxis was administered (4000 IU enoxaparin by subcutaneous injection) starting at 8 pm on the day of surgery and continuing for 15 days. Patients underwent spinal anesthesia with 1% hyperbaric marcaine and a tourniquet was used. Either bone-patellar tendon-bone (BPTP) or hamstring tendons (HTs) were harvested. All patients received post-operative cryotherapy and analgesia (60 mg ketorolac and 100 mg tramadol by infusion syringe-pump for 24 hours).

An articulated brace locked in extension was prescribed for 1 week. The brace was unlocked allowing 0°-90° range of motion on 7$^{th}$ post-operative day and maintained for 15 days. Joint evacuation was performed

on 7th post-operative day following brace unlocking if effusion (assessed by ballotment test) inhibited active quadriceps contraction (15-16). Effusion not inhibiting active quadriceps contraction was not recorded at 15th and 30th day follow-up. All patients performed isometric strengthening of the quadriceps femuris and active-passive knee motion on 1st and 7th postoperative day respectively. Crutch-assisted ambulation with full weight-bearing was allowed on 1st post-operative day. Suture stitches were removed at 15 days and the wound was inspected at 1 month. Follow-up visits were scheduled at 3 and 6 months for assessing progress in rehabilitation and final return to sports activities respectively.

Physical findings recorded pre- and post-operatively on 1st, 3rd, 7th, 15th, and 30th day for the purposes of this study included skin body temperature (SBT) as measured for 10 minutes by standard mercury thermometer at 6 pm and knee pain (KP) as measured by a visual analogue score (VAS). The volume of knee joint aspirates was recorded when a joint evacuation was performed on 7th post-operative day. WBC count, ESR and CRP values were collected pre-operatively and post-operatively on 1st, 3rd, 7th, 15th, and 30th day. Reference ranges in our laboratory are 4200-10,0000 cell/mm$^3$ for WBC count; < 15 mm/hour for adult men younger than 50 years and < 20 mm/hour for adult women younger than 50 years (1st hour) for ESR; ≤ 0.6 mg/dl for CRP. ESR measures the rate in mm per hour at which red blood cells settle in a test tube in a given time interval (1 or 2 hours) after mixing venous whole blood and sodium citrate anticoagulant-diluent solution. ESR is elevated when a local or systemic inflammation is present regardless of its cause. CRP is an acute-phase plasma protein increasing in bacterial infection and extensive tissue damage (17-18). It is analysed in venous blood collected in a serum-separating tube according to various methods. In the present study the measurement method is based on immunoprecipitation enhanced by polyethylene glycol at 340 nm. Specific antiserum is added in excess to buffered samples. The increase in absorbance caused by immunoprecipitation is recorded when the reaction has reached its end-point. The change in absorbance is proportional to the amount of antigen (CRP) in solution (Konelab™ analyzer, Thermo Electron OY, Vantaa, Finland).

The goal of data analysis was outlining the trend of changes from baseline to 30th post-operative for each of the following variables: SBT, KP, WBC count, ESR and CRP in the whole sample and in patients reconstructed with either BPTP or HTs. The 1st, 3rd and 7th post-operative days were selected as time-points for data collection because they yielded variations from baseline immediately after surgery, on the day of hospital discharge and on the day of re-admission for brace unlocking/joint motion respectively; the 15th and 30th days were chosen as the same time-points were used in another study investigating ESR and CRP changes after ACL reconstruction (11). In order to keep multiple comparisons to a minimum, *a priori* it was established to compare the post-operative changes of the index variables at each time-point with baseline values in the whole sample, and in the BPTP and HT subgroups. Follow-up at 6 month was considered adequate to rule out the onset of surgery-related septic arthritis.

*Statistical analysis*

Collected data were tabulated as mean ± standard deviation (SD) for SBT, WBC count, ESR and CRP. Median, lower and upper quartile were used for KP. The presence of outliers was determined using boxplots with fence range (mean ± SD and median). As no assumptions of normality were made, a non-parametric test (Friedman test and *a priori* multiple comparisons) was used for analysing the significance of post-operative changes of the index variables compared to baseline. The significance level was preset at $P < 0.05$. Statistical analysis was performed using a dedicated software (StatsDirect, version 2.6.1, StatsDirect Ltd, Cheshire, UK).

## RESULTS

A total of 58 patients were consecutively enrolled in the study. There were 46 males and 12 females (age range 15-52, median age 25). Thirty-five patients were IKDC class B and twenty-three class C. Meniscal tears were present in 27 patients and focal chondral lesions in 12 patients. Eight patients had associated meniscal and chondral lesions. Mean pre-operative TAT and SSD were 10 ± 3 SD mm and 5 ± 3 SD mm respectively. The ACL was reconstructed using BPTP in 13 patients and HTs in 45 patients. Joint effusion requiring aspiration was observed on 7th post-operative day in 30 out of 58 patients. Wound healing was uncomplicated in all patients as observed at stitch removal. All patients returned to 3 and 6 month follow-up and no clinical signs of infection were detected.

Mean SBT was significantly elevated on 1st, 3rd and 7th post-operative day and returned to values not differing from baseline on 15th and 30th day. SBT outlier values were observed on 1st (1 patient with 38°C), 3rd (2 patients with 37.6°C) and 7th day (2 patients with 38° and 38.4°C). Outlier values were observed in different patients at each time-point. Median KP showed a significant increase compared

**Table I.** *Pre- and post-operative variations of skin body temperature, knee pain, white blood cell, erythrocyte sedimentation rate and c-reactive protein in patients undergoing ACL arthroscopic reconstruction.*

| | Pre-op | 1st post-op day | 3rd post-op day | 7th post-op day | 15th post-op day | 30th post-op day | |
|---|---|---|---|---|---|---|---|
| [a]SBT (°Celsius) | | | | | | | |
| Mean | 36.6 | 37.2 | 36.7 | 36.8 | 36.6 | 36.6 | [#]P < 0.0001 |
| SD | 0.2 | 0.4 | 0.3 | 0.4 | 0.2 | 0.1 | |
| | | [§]P < 0.0001 | [§]P = 0.0153 | [§]P = 0.0377 | [§]P = 0.9766 | [§]P = 0.9766 | |
| [b]KP ([c]VAS) | | | | | | | |
| Median | 0 | 4 | 4 | 3 | 3 | 3 | [#]P < 0.0001 |
| Lower quartile | 0 | 3 | 3 | 3 | 3 | 2 | |
| Upper quartine | 2 | 5 | 5 | 4 | 4 | 4 | |
| | | [§]P < 0.0001 | [§]P < 0.0001 | [§]P < 0.0001 | [§]P < 0.0001 | [§]P < 0.0001 | |
| [d]WBC count ($10^3$ cells/$mm^3$) | | | | | | | |
| Mean | 6.66 | 8.68 | 6.85 | 7.70 | 7.22 | 6.34 | [#]P < 0.0001 |
| Standard deviation | 1.48 | 1.98 | 1.26 | 2.28 | 3.82 | 1.45 | |
| | | [§]P < 0.0001 | [§]P = 0.2787 | [§]P < 0.0001 | [§]P = 0.0005 | [§]P = 0.4957 | |
| [e]ESR (mm/hour) | | | | | | | |
| Mean | 5.02 | 9.71 | 27.10 | 38.81 | 24.67 | 11.36 | [#]P < 0.0001 |
| Standard deviation | 3.24 | 5.08 | 14.43 | 21.83 | 15.13 | 6.56 | |
| | | [§]P < 0.0001 | [§]P < 0.0001 | [§]P < 0.0001 | [§]P < 0.0001 | [§]P < 0.0001 | |
| [f]CRP (mg/dl) | | | | | | | |
| Mean | ≤ 0.60[g] | 1.78 | 3.73 | 2.32 | 0.74 | 0.61 | [#]P < 0.0001 |
| Standard deviation | | 1.26 | 2.71 | 1.99 | 0.50 | 0.08 | |
| | | [§]P < 0.0001 | [§]P < 0.0001 | [§]P < 0.0001 | [§]P = 0.1837 | [§]P > 0.9268 | |

*[#]P: Friedman test; [§]P: multiple comparisons of post-operative changes with pre-operative values; [a]SBT: skin body temperature; [b]KP: knee pain [c]VAS: visual analogue score; [d]WBC: white blood cell; [e]ESR: erythrocyte sedimentation rate; [f]CRP: C-reactive protein; [g]0.6: lowest detectable concentration rate.*

to baseline values throughout the post-operative time.

Mean WBC count significantly increased on 1st, 7th and 15th day while no difference from baseline was observed on 3rd and 30th post-operative day. The following outliers were recorded from 1st to 30th post-operative day: 3 patients on 1st day ($12.82\times10^3$ cell/$mm^3$, $13.79\times10^3$ cell/$mm^3$ and $14.79\times10^3$ cell/$mm^3$), 2 patients on 3rd day ($9.72\times10^3$ cell/$mm^3$ and $10.51\times10^3$ cell/$mm^3$), 1 patient on 7th day ($14.90\times10^3$ cell/$mm^3$), 2 patients on 15th day ($11.74\times10^3$ cell/$mm^3$ and $15.12\times10^3$ cell/$mm^3$) and 3 patients on 30th day ($9.44\times10^3$ cell/$mm^3$, $9.63\times10^3$ cell/$mm^3$ and $9.98\times10^3$ cell/$mm^3$). All three patients with an outlier WBC count on 1st day showed outlier counts at some time-point between the 3rd and 30th day.

Mean ESR was significantly elevated on 1st post-operative day , increased on 3rd day and peaked on 7th day. ESR decreased on 15th and 30th day but remained significantly higher than baseline. The following outliers were recorded from 1st to 30th post-operative day: 1 patients on 1st day (22 mm/hour), 1 patients on 3rd day (75 mm/hour), 1 patient on 7th day (87 mm/hour), 2 patients on 15th day (77 mm/hour and 62 mm/hour) and 1 patients on 30th day (37 mm/hour). Outlier values were observed in different patients at each time-point except for one patient who showed outlier values on 7th and 15th day.

Mean CRP significantly increased on 1st post-operative day, peaked on 3rd day and decreased on 7th day. Levels on 15th and 30th day did not differ from baseline. Outliers recorded from 1st to 30th post-operative day were as follows: 3 patients on 1st day (4.9 mg/dl, 5.2 mg/dl and 5.4 mg/dl), 2 patients on

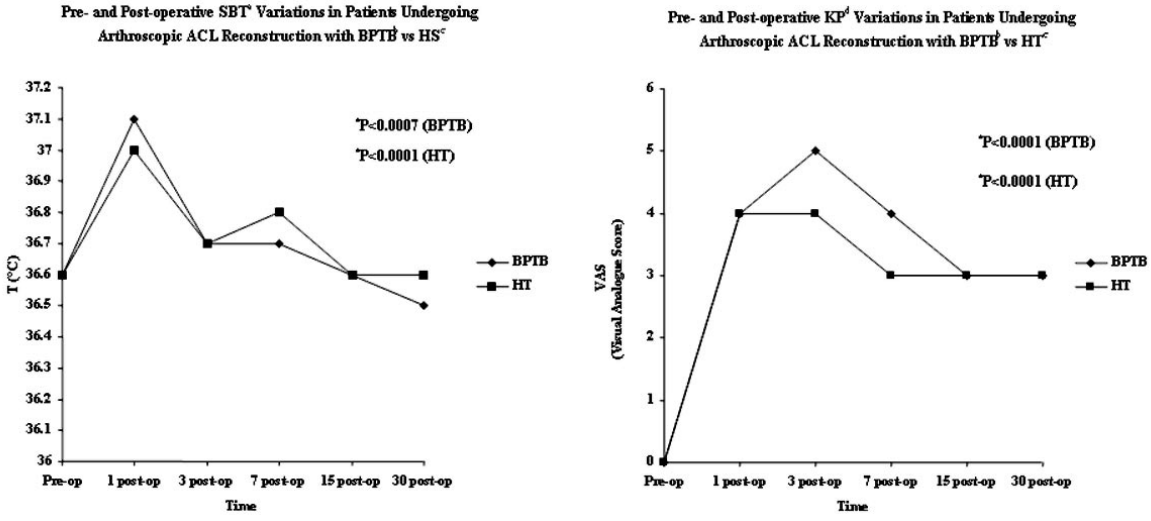


**Fig. 1**. *Pre- and post-operative variations of SBT[a] (Skin Body Temperature, Figure 1A on the left) and KP[d] (Knee Pain, Figure 1B on the right) in patients undergoing arthroscopic ACL reconstruction with BPTB[b] (bone-patellar tendon-bone) versus HT[c] (hamstring tendon). A similar trend in SBT and KP post-operative variations can be observed in patients receiving either BPTB and HT, although the magnitude of SBT and KP changes is higher in patients reconstructed with BPTB which is associated with a more extensive tissue manipulation. [*]P: statistical significance (Friedman test) of comparison between baseline and post-operative variables stratified by type of autograft.*

**Table II.** *Pre- and post-operative laboratory test variations in patients undergoing ACL arthroscopic reconstruction with bone- Patellar Tendon Bone (BPTP) versus Hamstring Tendons (HTs).*

| | Pre-op | 1st post-op day | 3rd post-op day | 7th post-op day | 15th post-op day | 30th post-op day | #P |
|---|---|---|---|---|---|---|---|
| **[a]WBC count** ($10^3$ cells/$mm^3$) | | | | | | | |
| Mean ± SD (BPTP n=13) | 7.24±1.93 | 9.89±1.96 | 7.75±1.28 | 8.26±2.63 | 7.95±2.13 | 6.63±1.99 | < 0.0001 |
| | | *P < 0.0001 | *P = 0.1949 | *P = 0.0216 | *P = 0.0402 | *P = 0.1949 | |
| Mean ± SD (HTs n=45) | 6.51±1.35 | 8.33±1.87 | 6.59±1.14 | 7.54±2.18 | 7.01±1.87 | 6.25±1.27 | < 0.0001 |
| | | *P < 0.0001 | *P = 0.5976 | *P < 0.0001 | *P = 0.0042 | *P = 0.9439 | |
| **[b]ESR** (mm/hour) | | | | | | | |
| Mean ± SD (BPTP n=13) | 2.77±1.48 | 9.00±4.67 | 38.46±15.76 | 50.85±21.12 | 30.15±16.80 | 9.23±3.85 | < 0.0001 |
| | | *P < 0.0001 | *P < 0.0001 | *P < 0.0001 | *P < 0.0001 | *P < 0.0001 | |
| Mean ± SD (HTs n=45) | 5.67±3.32 | 9.91±5.23 | 23.82±12.36 | 35.33±20.99 | 23.09±14.43 | 11.08-7.09 | < 0.0001 |
| | | *P < 0.0001 | *P < 0.0001 | *P < 0.0001 | *P < 0.0001 | *P < 0.0001 | |
| **[c]CRP** (mg/dl) | | | | | | | |
| Mean ± SD (BPTP n=13) | ≤ 0.60[d] | 2.38±1.55 | 6.47±2.44 | 2.99±2.38 | 0.74±0.50 | ≤ 0.60 [d] | < 0.0001 |
| | | *P < 0.0001 | *P < 0.0001 | *P < 0.0001 | *P = 0.5943 | *P >0.9999 | |
| Mean ± SD (HTs n=45) | ≤ 0.60[d] | 1.60±1.12 | 2.94±2.24 | 2.13±1.85 | 0.74±0.51 | 0.61±0.09 | < 0.0001 |
| | | *P < 0.0001 | *P < 0.0001 | *P < 0.0001 | *P = 0.2248 | *P = 0.9193 | |

*[a]WBC: white blood cell; [b]ESR: erythrocyte sedimentation rate; [c]CRP: C-reactive protein; [d]0.6: lowest detectable concentration rate; [#]P: Friedman test; [*]P: multiple comparisons of post-operative changes with pre-operative values*

**Table III.** *Physical findings and laboratory tests in patients developing septic arthritis after arthroscopic acl reconstruction reported in published papers*

| Author | Number of infected patients | Type of graft | Average time to presentation (days from surgery) | Average time to diagnosis | [a]SBT (° Celsius) | [b]KP at wound | Effusion/ Swelling | Average [c]WCB count ($10^3$ cell/$mm^3$) | Average [d]ESR (mm/hour) | Average [e]CRP (mg/dl) | Result of aspiration culture |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **McAllister et al.**[8] (1999) | 4/831 (0.48%) | Autograft | 11.25 | 24 hours within presentation | 39 (average) | Yes (4/4) | Yes (4/4) | 9.74 | 79 | 26 | Positive (all patients) |
| **Indelli et al.**[5] (2002) | 5/3500 (0.14%) plus 1 patient referred | 4 Autografts 2 Allografts | 20 | 7.5 days from onset of symptoms to surgery for infection | [#]N/R | [#]N/R | [#]N/R | [#]N/R | [#]N/R | [#]N/R | Positive (all patients) |
| **[§]Burks et al.**[9] (2003) | [§]8/1918 (0.42%) | Autograft | 19 | N/R | [#]N/R | Yes (4/4) | Yes (4/4) | 8.4 | 48 | [#]N/R | Positive (all patients) |
| **Scholling-Borg et al.**[6] (2003) | 10/575 (1.7%) | Autograft | 9.5 | 15.4 days from surgery | 37.6-39-6 (range) | Yes (6/10) | Yes (10/10) | 8.4 | 62.3 | 9.18 | Positive (8/10 patients) |
| **Judd et al.**[7] (2006) | 11/1615 (0.68%) | Autograft | 14.2 | On day of presentation (8 patients) 2 days after presentation (2 patients) 5 days after presentation (1 patient) | Yes (5/11) Temperature values N/R | Yes (8/11) | Yes (11/11) | 9.8 | 67 | 7.7 | Positive (all patients) |

*[a]SBT: skin body temperature; [b]KP: knee pain; [c]WCB count: white blood cell count; [d]ESR: erythrocyte sedimentation rate; [e]CRP: C-reactive protein; [#]N/R: not reported; [§]: the results reported by Burks et al. refer to only 4 of the 8 infected patients (those treated with early reimplantation of the graft).*

3rd day (10.2 mg/dl and 11.2 mg/dl), 3 patient on 7th day (8.8 mg/dl, 6.7 mg/dl and 6.4 mg/dl), 3 patients on 15th day (3.2 mg/dl, 2.8 mg/dl and 2.4 mg/dl) and 1 patient on 30th day (1.2 mg/dl). Outlier values were observed in different patients at each time-point.

The post-operative changes and significance of statistical testing of SBT, KP, WBC count, ESR and CRP are reported in Table I. The SBT and KP variations, and the WBC count, ESR and CRP changes stratified in relation to the type of autograft used for reconstruction showed that the trend of post-operative changes paralleled that observed in the whole sample, as shown in Fig. 1 and Table II respectively.

## DISCUSSION

The diagnosis of septic arthritis related to ACL surgery can only be achieved when pathogens are detected in aspirates (5-9), although false-negative testing has been described in patients who were administered non-targeted antibiotics (6). However, the potential for joint contamination recommends aspiration should not be used as a screening procedure. Knowledge of the trend of clinical and laboratory indicators in infection-free patients may assist clinicians when septic arthritis is suspected, pending definitive confirmation by joint aspirate. In our series KP was observed throughout the first month. Since gender-related, as well as socio-cultural factors, affect pain perception (19), our results should be interpreted with caution. They suggest that post-operative KP may be part of an uncomplicated course after ACL surgery. KP at wound has been reported in some but not in all patients developing septic arthritis (Table III).

Effusion hindering quadricep contraction on 7th post-operative day occurred in 52% of our patients. We regard joint effusion as clinically meaningful only when it inhibits quadricep contraction. Thus, the criterion and modality used to record effusion may have underestimated its actual occurrence. Swelling and effusion have always been associated with septic arthritis in published series (Table III).

The SBT increase observed during the first post-operative week may be triggered by surgical trauma and inception of 0-90° passive motion. In our practice we closely monitor patients showing outlier SBT values, particularly if they occur 1 week after surgery. Published series have inconstantly reported the presence of fever in infected patients (Table III).

ACL reconstruction seems to boost an immune response (20) characterised by elevation of WBC count on 1st post-operative day, decreasing on 3rd day and newly increasing from 7th to 15th day. The observed trend of WBC count would accordingly recommend clinical surveillance when WBC levels do not go back to normality 2 weeks after surgery. However, some authors have reported that mean WBC counts are not markedly elevated even in cases of septic arthritis (Table III).

ESR markedly increased during the first week then gradually decreased without returning to

normality 1 month after surgery. CRP levels peaked on 3rd day and returned to normal within 2 weeks. The ESR and CRP temporal trend observed in our series would seem to characterise an uncomplicated course following ACL-reconstruction. In fact, similar findings were reported in another study investigating ESR and CRP changes in 45 chronically ACL-insufficient patients −with no associated meniscal and/or cartilage lesions undergoing reconstruction with BPTP (11). In our series the type of graft did not seem to affect the trend of post-operative WBC count, ESR and CRP levels. Markedly elevated ESR and CRP levels collected at the time-point of presentation were observed in infected patients in published series (Table III). Interestingly, one paper describing ESR and CRP changes in elective orthopaedic surgery (21) showed that CRP levels increase on 2nd (total knee arthroplasty and lumbar microdiscectomy) and on 3rd (total hip arthroplasty) post-operative day, usually returning to normal within 21 days. Conversely, ESR levels exhibited a slower and more irregular decrease. The time interval for return to normality of CRP levels would consequently vary in relation to the type of surgery. In the case of arthroscopic ACL reconstruction, CRP levels decrease after 1 week and go back to normality within 2 weeks in most patients.

Three limitations of our study should be acknowledged. Firstly, the lack of infected controls did not allow to assess the role of the examined variables as diagnostic predictors. However, such a study would be difficult to conduct on large samples considering the relative rarity of septic arthritis after ACL reconstruction. Secondly, outliers were observed at different time-points. Care was devoted to scrupulous data collection and recording yet we cannot entirely rule out that errors may have occurred. Since lack of published reference values prevents from discriminating which outliers represent errors or an actual enhanced response in some individuals, outliers were not discarded opting for a non-parametric test, which is least influenced by outliers, for data analysis. Lastly, since our study focused on CRP changes, the overall status of the inflammation response following ACL surgery has not been exhaustively described (17-18, 22-23).

In conclusion, the results of our study and a comparison with published data would suggest that clinical surveillance may be advisable when SBT, occurrence of joint effusion and CRP levels deviate from the described reference trend two weeks after surgery. In these circumstances, suspicion of septic arthritis warrants aspiration and culturing in order to avert a diagnostic delay.

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