Protective effects of camel milk against pathogenicity induced by *Escherichia coli* and *Staphylococcus aureus* in Wistar rats

MOHAMED MOHAMED SOLIMAN1,2, MAGDY YASSIN HASSAN3,4, SALAMA ABDEL-HAFIZ MOSTAFA3,5, HUSSEIN ABDEL-MAKSOUS ALI2 and OSAMA MOSEILHY SALEH6,7

1Medical Laboratory Department, Faculty of Applied Medical Sciences, Turabah, Taif University, Taif 21944, Saudi Arabia; 2Department of Biochemistry, Faculty of Veterinary Medicine, Benha University, Benha, Qalyubia 13511, Egypt; 3Department of Medical Microbiology, Faculty of Applied Medical Sciences, Turabah, Taif University, Taif 21974, Saudi Arabia; 4Department of Reproductive Diseases; 5Immunity Unit, Animal Reproduction Research Institute, Giza, Al-Haram 12557, Egypt; 6Department of Medical Biotechnology, Faculty of Applied Medical Sciences, Turabah, Taif University, Taif 21974, Saudi Arabia; 7Natural Products Research Department, National Center for Radiation Research and Technology, Giza, Cairo 11787, Egypt

Received December 19, 2014; Accepted September 22, 2015

DOI: 10.3892/mmr.2015.4486

Abstract. The aim of the present study was to investigate the protective effects of camel milk on hepatic pathogenicity induced by experimental infection with *Escherichia (E. coli)* and *Staphylococcus aureus (S. aureus)* in Wistar rats. The rats were divided into six groups: The control and camel milk groups received water and camel milk, respectively; two groups received camel milk for 2 weeks prior to intraperitoneal injection of either *E. coli* or *S. aureus*; and two groups were injected intraperitoneally with *E. coli* and *S. aureus*, respectively. All animals were maintained under observation for 7 days prior to biochemical and gene expression analyses. The rats treated with camel milk alone exhibited no changes in expression levels of glutamic-pyruvate transaminase (GPT) or glutamic-oxaloacetic transaminase (GOT), compared with the water-treated group. The *E. coli* - and *S. aureus*-injected rats exhibited a significant increase in oxidative stress, and prior treatment with camel milk normalized the observed changes in the expression levels of GPT, GOT and malondialdehyde (MDA). Treatment with camel milk decreased the total bacterial count in liver tissue samples obtained from the rats injected with *E. coli* and *S. aureus*. Camel milk administration increased the expression levels of glutathione-S-transferase and superoxide dismutase, which were downregulated following *E. coli* and *S. aureus* injection. In addition, camel milk downregulated the increased expression of interleukin-6 and apoptosis-associated genes. Of note, administration of camel milk alone increased the expression levels of the B cell lymphoma 2-associated X protein and survivin anti-apoptotic genes, and supplementation prior to the injection of *E. coli* and *S. aureus* induced further upregulation. In conclusion, camel milk exerted protective effects against *E. coli* and *S. aureus* pathogenicity, by modulating the extent of lipid peroxidation, together with the antioxidant defense system, immune cytokines, apoptosis and the expression of anti-apoptotic genes in the liver of Wistar rats.

Introduction

Camels (*Camelus romedarius*) are important to the lifestyle of several communities, particularly those of the Middle East. Furthermore, camels contribute to the economy and food security of humans by providing milk and meat. It is well-established that milk is a source of energy, proteins, vitamins and minerals. In addition to its value as a nutrient source, milk also has antibiotic properties. The milk of mammals is protected to various extents against microbial contamination by natural inhibitory systems, including lactoferrins, lysozymes, immunoglobulins and free fatty acids (1,2). Camel milk is reported to have a more marked inhibitory system, compared with cow milk (1). Notably, the levels of lysozyme and lactoferrins in camel milk are two and three times higher than those of cow milk, respectively (2). Camel milk contains peptides and proteins, which exhibit biological activities that have beneficial effects on several bioprocesses, including digestion, absorption, growth and immunity (3,4). Furthermore, camel milk can be stored at room temperature for longer periods of time, compared with the milk from other animals (5).

Camel milk is used in the treatment of autoimmune diseases, dropsy, jaundice, splenomegaly, tuberculosis, asthma, anemia, piles, diabetes and as an antimicrobial (6). In addition, camel
milk has antitoxic effects against cadmium chloride (7,8), carbon tetrachloride (9), cisplatin (10) and paracetamol (11). Camel whey proteins assist in the prevention of several human diseases (12), and dietary whey supplements may improve wound healing by increasing glutathione-S-transferase synthesis and cellular anti-oxidant defense (13). The liver is an important organ exposed to pathogenicity during microbial infection (7). Camel milk, but not bovine milk, significantly inhibits HepG2 and MCF7 cell proliferation through activation of the mRNA expression and activity of caspase-3 (14). Furthermore, camel milk increases the expression levels of oxidative stress markers, including heme oxygenase-1, and increases the production of reactive oxygen species in the two cells (14). Camel milk lysozyme has bactericidal effects against gram-positive bacterial strains and exerts bactericidal effects against gram-negative strains (1).

*Staphylococcus aureus* (*S. aureus*) is a gram-positive bacteria, which causes numerous infections in humans and animals (15). *S. aureus* can survive for hours to weeks, and even months, on dry environmental surfaces (15-17). Similar to *S. aureus*, *Escherichia coli* (*E. coli*) is a gram-negative microorganism, which causes severe pathogenicity to the infected host. It has been reported that camel milk exhibits bacteriostatic effects against *E. coli* and *Listeria monocytogenes* (18). Camel milk is also considered to have medicinal properties against certain pathogens in the Middle East (1,2). Therefore, the aim of the present study was to examine the protective effects of camel milk against *E. coli* and *S. aureus*-induced hepatic pathogenicity in Wistar rats.

**Materials and methods**

**Materials and bacterial strains.** *E. coli* and *S. aureus* strains were obtained from Animal Reproduction Research Institute (Alharam Giza, Egypt). QIAzol for RNA extraction and oligo dT primers were purchased from Qiagen, Inc. (Valencia, CA, USA). Wistar rats were purchased from the King Fahd Institute for Scientific Research, King AbdulAziz University, Jeddah, Saudi Arabia). Solvents and associated materials were obtained from ADWIA Pharmaceutical Co. (El Oubor, Egypt). The primers for gene expression analysis were purchased from Macrogen, Inc., (Seoul, Republic of Korea). The DNA ladder was purchased from MBI, Fermentas (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The biochemical kit for malondialdehyde (MDA) was purchased from Bio Diagnostic Company (Dokki, Egypt). Camel milk, collected from healthy, disease-free Magrabi females (5-10 years old) of the Magrabi breed, was provided daily from farms in Turabah (Taif, Saudi-Arabia). All animal procedures were approved by the Ethical Committee Office of the Dean of Scientific Affairs of Taif University (Taif, Saudi Arabia).

**Camel milk preparation.** Camel milk samples were collected daily, early in the morning, from a camel farm in Turabah, Saudi-Arabia. The milk was collected from a healthy 4 year-old camel by hand into sterile screw bottles, and maintained in cool boxes until transported to the laboratory. The rats were supplemented with unpasteurized camel milk, which was administered orally at a dose of 100 ml/24 h/cage (six rats), based on a previous study by Althnaian *et al* (19) at a fixed time of 9.00 am.

**E. coli preparation.** The *E. coli* strains were isolated from cases of bovine mastitis grown in brain/heart infusion broth. When the bacteria were in the logarithmic phase of growth, the suspension was centrifuged at 15,000 x g for 15 min (Animal Reproduction Research Institute), the supernatant was discarded, and the bacteria were re-suspended and diluted in sterile saline (1:1). The rats were injected intraperitoneally with 1 ml saline containing 2x10⁸ colony forming units (CFU) of *E. coli*. Immediately following bacterial challenge, the rats were maintained under observation for 7 days.

**S. aureus preparation.** Preliminary confirmation and phenotypic investigations were performed, according to standard protocols (15), using gram staining and biochemical parameters, including a coagulase test, and were screened by growth on Baird-Parker selective agar. Following confirmation, the bacterial culture was cultured in tryptic broth and incubated overnight. The bacterial culture was then centrifuged at 15,000 x g for 15 min, and the pellet was resuspended and washed with sterile phosphate-buffered saline (PBS). The viable bacterial count was adjusted to ~1x10⁶ CFU/ml. Serial dilution was performed in PBS to obtain a final concentration of 5x10⁹/0.1 ml bacterial suspension.

**Inoculation of E. coli and S. aureus strains into rats and experimental design.** A total of 60 male Wistar rats (4-week-old; 80-100 g) were selected randomly. The rats were exposed to a 12 h light/dark cycle and provided with access to food and water *ad libitum*. The 60 rats were divided into six groups (10 rats/group) with five rats per cage. The control group was fed a normal diet; the camel milk group was administered with a dose of 100 ml camel milk per six rats, based on a previous study (19); the *E. coli* group was intraperitoneally injected with a virulent strain of *E. coli* at a dose of 2x10⁹ CFU/ml/rat (20); the *E. coli* + camel milk group was administered with *E. coli*, as in the *E. coli* group following camel milk supplementation; the *S. aureus* group was intraperitoneally injected with a virulent strain of *S. aureus* at a dose of 1x10⁸ CFU/ml/rat (21); and the *S. aureus* + camel milk group was treated in the same way as the *S. aureus* group, following prior camel milk supplementation. The rats in the *E. coli* or *S. aureus* + camel milk groups were pre-administered with camel milk for 2 weeks prior to pathogen injection. All animals were maintained under observation for 7 days. At the end of the experimental period (day 8), the rats were sacrificed by decapitation following overnight fasting and diethyl ether inhalation. Blood samples (5-8 ml/rat) were obtained for serum extraction by centrifugation at 1,000 x g for 10 min at room temperature, and liver samples were removed and placed under aseptic conditions in QIAzol reagent for RNA extraction and gene expression analyses, and in sterile tubes for total bacterial count.

**Serum MDA measurements.** Serum MDA, GPT and GOT were measured using a commercially available kit prior to spectrophotometric analysis. The activities of MDA were determined using an ELISA reader at an optical density (OD) of 532 nm (Absorbance Microplate Reader ELx 800TM BioTek®, BioTek Instruments, Seattle, WA, USA). For liver biomarkers, Serum levels of GPT and GOT were measured spectrophotometrically using specific commercial kits (Biodiagnostic Company,
Reverse transcription- quantitative polymerase chain reaction (RT-qPCR) analysis of gene expression levels. 
Liver tissues were collected from the rats, flash frozen in 1 ml QIAzol reagent and subsequently stored at -70˚C. The frozen samples (50-100 mg) were then homogenized using a Polytron 300 D homogenizer (Lauda-Brinkmann, Delran, NJ, USA). Total RNA was extracted via chloroform extraction, followed by nucleic acid precipitation using isopropyl alcohol (absolute chloroform). The pellet was washed with 70% ethanol and re-suspended in molecular biological grade water (absolute nanopure water).

The RNA (2 µg) was incubated at 65˚C for 10 min and was then reverse transcribed using 100 units of Moloney murine leukemia virus reverse transcriptase (Gibco; Thermo Fisher Scientific, Inc.), 50 pmol of poly (dT) primer and 20 nmol dNTPs, in a total volume of 11 µl at 37˚C for 1 h. Following heating at 94˚C for 5 min, PCR amplification was performed with 2.5 units Taq polymerase (PerkinElmer, Inc., Waltham, MA, USA), 3 mM MgCl₂ and 50 pmol of the forward and reverse primers specific for the respective genes, in a total volume of 25 µl. The PCR conditions of the genes analyzed are listed in Table I. The thermocycling conditions were as follows: Each cycle consisted of denaturation at 94˚C for 1 min, annealing at the gene-specific temperatures for each gene (Table I) for 1 min, extension at 72˚C for 1 min and final extension at 72˚C for 7 min. The RT-qPCR products were visualized under an ultraviolet lamp by electrophoresis in 1.5% agarose gel stained with ethidium bromide. The intensities of the bands were analyzed densitometrically using the NIG Image program (http://rsb.info.nih.gov/nih-image/).

Statistical analysis. The data are expressed as the mean ± standard error of the mean from three independent experiments per treatment. *P<0.05, vs. control and CM milk groups; †P<0.05, vs. E. coli group; ‡P<0.05, vs. S. aureus group. GPT, glutamate pyruvate transaminase; GOT, glutamate oxalate transaminase; E. coli, Escherichia coli; S. aureus, Staphylococcus aureus; CM, camel milk.

Table I. Primer sequences and polymerase chain reaction conditions of the genes analyzed.

<table>
<thead>
<tr>
<th>mRNA (bp)</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
<th>Cycles (n)</th>
<th>Annealing temp (˚C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-3 (282)</td>
<td>ACGGTACCGCGAAGAAAGTGAC</td>
<td>TCCTGATCTTCGATTTCAAGGC</td>
<td>30</td>
<td>52</td>
</tr>
<tr>
<td>Survivin (390)</td>
<td>CTGATTTGGCCCATGTGTTTT</td>
<td>TCATCTGACGCTCCGTTTTC</td>
<td>35</td>
<td>52</td>
</tr>
<tr>
<td>Bax (600)</td>
<td>GTCGTCAGAATCTCAAGTACAT</td>
<td>CACAGTGATATGACGATCATC</td>
<td>35</td>
<td>58</td>
</tr>
<tr>
<td>TGF-β1 (456)</td>
<td>TGAGTGGCTGCTTTTGAGG</td>
<td>TGGTTTAGAGGGCAAGGAC</td>
<td>35</td>
<td>60</td>
</tr>
<tr>
<td>IL-6 (450)</td>
<td>AGTTGCTTTGCTGGGATCTGATGT</td>
<td>TGGCTCTGAAATGCCTGCTGTTTG</td>
<td>35</td>
<td>58</td>
</tr>
<tr>
<td>GST (575)</td>
<td>GTGGAGTGGAGTTGGAAGAA</td>
<td>GTCTGGCCTCAGGTCACATAG</td>
<td>35</td>
<td>55</td>
</tr>
<tr>
<td>SOD (410)</td>
<td>AGATTAACAGGAACGGA</td>
<td>TCTCACAGATTGAGGCAACG</td>
<td>33</td>
<td>55</td>
</tr>
<tr>
<td>GAPDH (309)</td>
<td>AGATCCACAAACGGATACATT</td>
<td>TCCCTCAAGATTGTCAGCAA</td>
<td>25</td>
<td>52</td>
</tr>
</tbody>
</table>

Table II. Protective effects of camel milk, determined by the expression levels of GPT and GOT in liver tissues, and the total E. coli and S. aureus count/g tissue 7 days following exposure to E. coli and S. aureus in Wistar rats.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Control</th>
<th>CM</th>
<th>E. coli</th>
<th>CM + E. coli</th>
<th>S. aureus</th>
<th>S. aureus + CM</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPT (U/l)</td>
<td>78±8.6</td>
<td>63±4.4</td>
<td>174±9.5</td>
<td>98.3±10.7</td>
<td>151.7±6.3</td>
<td>77±8</td>
</tr>
<tr>
<td>GOT (U/l)</td>
<td>62±7.2</td>
<td>64±4.9</td>
<td>145±5.5</td>
<td>84.3±2.9</td>
<td>153±9.3</td>
<td>76±12.5</td>
</tr>
<tr>
<td>Total E. coli count</td>
<td>-</td>
<td>-</td>
<td>4.5x10⁵</td>
<td>3.4x10⁵</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total S. aureus count</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7x10⁵</td>
<td>3.6x10⁵</td>
</tr>
</tbody>
</table>

Values are expressed as means ± standard error of the mean from three independent experiments per treatment. *P<0.05, vs. control and CM milk groups; †P<0.05, vs. E. coli group; ‡P<0.05, vs. S. aureus group. GPT, glutamate pyruvate transaminase; GOT, glutamate oxalate transaminase; E. coli, Escherichia coli; S. aureus, Staphylococcus aureus; CM, camel milk.
injection with *E. coli* and *S. aureus*. The injection of *E. coli* and *S. aureus* induced significant increases in the expression levels of GPT and GOT due to the hepatic pathogenicity of the bacterial strains. Prior supplementation with camel milk decreased the bacterial-induced upregulation in the expression levels of GPT and GOT. In addition, the total bacterial counts were higher in the liver tissues of the *E. coli* and *S. aureus*-injected rats, compared with the control and camel milk groups, and were significantly decreased in the pathogen injected rats supplemented with camel milk (Table II).

**Protective effects of camel milk on the survival rates of Wistar rats injected with *E. coli* and *S. aureus*.** The injection of *E. coli* and *S. aureus* led to mortality rates of 60 and 70%, respectively. Camel milk supplementation induced protective effects, and the survival rates in the *E. coli* and *S. aureus*-injected rats following camel milk administration were 80 and 70%, respectively (Table III). Notably, the percentage of camel milk protection from mortality in the *E. coli* and *S. aureus* rats was 40% (Table III).

**Protective effects of camel milk on *E. coli* and *S. aureus*-induced changes in serum MDA and hepatic antioxidant genes in Wistar rats.** As shown in Fig. 1A, injection with the *E. coli* and *S. aureus* strains induced significant increases in the expression levels of MDA, marker of oxidative stress. Camel milk supplementation decreased the expression levels of MDA following *E. coli* and *S. aureus* injection. By contrast, *E. coli* and *S. aureus* decreased the mRNA expression levels of glutathione-S-transferase (GST; Fig. 1B) and superoxide dismutase (SOD; Fig. 1C), and prior supplementation of camel milk normalized the decrease in the expression levels of GST and SOD. Camel milk alone increased the expression levels of GST and SOD, demonstrating its antioxidant action.

**Protective effects of camel milk on *E. coli* and *S. aureus*-induced changes in mRNA expression levels of transforming growth factor-β1 (TGF-β1) and interleukin-6 (IL-6) in Wistar rats.** As shown in Fig. 2A, camel milk upregulated the expression of TGF-β1. *E. coli* and *S. aureus* also upregulated the expression of TGF-β1. Supplementation with camel milk with either *E. coli* or *S. aureus* induced additive stimulatory effects on the expression of TGF-β1. However, camel milk did not affect the expression of IL-6, whereas the two pathogens upregulated the expression of IL-6 significantly. Supplementation with camel milk prior to *E. coli* and *S. aureus* injection downregulated the expression of IL-6.

**Protective effects of camel milk on *E. coli* and *S. aureus*-induced changes in mRNA expression of caspase-3 in Wistar rats.** To examine the effects of camel milk on the expression of caspase-3, RT-qPCR analysis of liver tissue samples was performed. As shown in Fig. 3, camel milk did not significantly alter the expression of caspase-3; however, *E. coli* and *S. aureus* significantly upregulated the expression of caspase-3 (Fig. 3). Supplementation with camel milk prior to *E. coli* or *S. aureus* injection significantly reduced the increased expression of caspase-3 induced by the pathogens.

**Protective effects of camel milk supplementation on *E. coli* and *S. aureus*-induced changes in the mRNA expression levels of B cell lymphoma 2-associated X protein (Bax) and survivin in Wistar rats.** As shown in Fig. 4A and B, camel milk upregulated the mRNA expression levels of Bax and survivin. *E. coli* and *S. aureus* also significantly increased the expression levels of Bax and survivin. Prior supplementation with camel milk resulted in additive stimulatory effects on the mRNA expression levels of Bax and survivin when injected with *E. coli* and *S. aureus* (Fig. 4A and B).

**Discussion**

The present study reported that camel milk supplementation reversed the increase in oxidative stress induced by *E. coli* and *S. aureus* infection. Furthermore, the two pathogens induced a decrease in the expression of antioxidants and affected the expression levels of inflammatory cytokines, apoptotic, pro-apoptotic and anti-apoptotic genes. These changes included normalization in the expression levels of antioxidants, caspase-3, IL-6 and TGF-β. It is well-established that *S. aureus* infections can spread through contact with pus from an infected wound, skin-to-skin contact with an infected person due to bacteria producing hyaluronidase that degrades tissues and contact with objects, including towels, sheets, clothing or athletic equipment, used by an infected individual (16). A large polysaccharide capsule protects the organism from recognition by the immune defenses in cows (15). *E. coli* is gram-negative microorganism, which causes severe pathogenicity to the infected host, and it has been reported that camel milk has a bacteriostatic effect against *E. coli* and *L. monocytogenes* (18).
Oxidative stress initiates apoptosis through mitochondrial stress caused by free radicals (23,24), which are indicated by levels of MDA. This involves a balance between pro-apoptotic and anti-apoptotic proteins, which enhance the permeability of the mitochondrial outer membrane for the release of caspase activators (25). Caspase-3 has been identified as an important contributor to apoptosis, in which activated caspase-3 causes the cell to undergo apoptosis through the cleavage of key cellular proteins, including cytoskeletal proteins, leading to the typical morphological changes observed in cells undergoing apoptosis (25,26), which is counteracted by camel milk supplementation.

Cytokines are low molecular weight proteins produced by several types of cell (27) and exhibit beneficial and pathological effects on target cells. Imbalanced expression of cytokines has been implicated in the progression of several diseases (28). During E. coli and S. aureus pathogenicity, increased expression levels of TGF-β1 and IL-6 were reported in the present study. The mRNA expression levels of IL-6 increased following E. coli and S. aureus injection, and prior
supplementation with camel milk normalized these increases in IL-6 and induced additive effect on TGF-β1 expression. TGF-β1 performs numerous cellular functions, including the control of cell growth, cell proliferation, cell differentiation and apoptosis (29). TGF-β1 can be regarded as an early mediator of the inflammatory response (30). TGF-β1 is one of the major pro-fibrogenic cytokines in various tissues, and is implicated in the etiology of pancreatic fibrosis, function of leukocyte chemotaxis, and fibroblast and smooth muscle cell mitogenesis (31-33). In the present study, camel milk regulated the expression levels of TGF-β1 and IL-6, thereby controlling the inflammation and apoptosis induced by E. coli and S. aureus injection. Previous studies have reported that camel milk is the most active milk against E. coli, S. aureus, Salmonella typhimurium and rotavirus (1,34). It has also been demonstrated that camel milk, in addition to secretory immunoglobulin (Ig)A and IgM, also contains numerous non-antibody components, which possess antiviral activity, including lactoferrin (34).

Apoptosis is an evolutionary conserved process by which organisms remove cells that are superfluous, have outlived their usefulness, or are dangerous for the survival of the organism (35). The apoptotic process can occur intracellularly, involving the release of several factors, including caspase 3 and 6 from mitochondria, which can be activated by various stressors, and pro-apoptotic proteins, including Bax, which migrate from the inter-membrane space of the mitochondria into the cytosol to act as sensors of cell damage or stress (35,36).

During infection, cytochrome c binds the adaptor protein, apoptotic protease-activating factor-1, forming a large multi-protein structure known as the apoptosome (25). The apoptosome then recruits and activates caspase-9, which in turn activates downstream effector caspases, including caspases-3 and 7, leading to apoptosis (25). Under normal conditions, caspase activity is controlled by a protein family known as inhibitor of apoptosis proteins, among which is survivin (37). Anti-apoptotic Bcl-2 and Bcl-xL proteins act to prevent permeabilization of the mitochondrial outer membrane by inhibiting the action of pro-apoptotic Bax, a cytosolic protein, located in the mitochondrial membrane (38). It has been reported that caspase-3 inhibits reactive oxygen species production, and is required for efficient execution of apoptosis (39). The survivin protein acts to inhibit caspase activation, thereby leading to negative regulation of apoptosis and/or programmed cell death (40), which was concordant with the results of the present study. The present study demonstrated that camel milk upregulated the gene expression of pro-apoptotic Bax, in order to control and regulate the gene expression of anti-apoptotic.
survivin. Camel milk exhibited beneficial effects when supplemented during *E. coli* and *S. aureus* infection.

In conclusion, the present study demonstrated that camel milk had protective effects against pathogenicity induced by *E. coli* and *S. aureus* in Wistar rats. The protective effects occurred through the regulation of antioxidant genes, genes associated with apoptosis/anti-apoptosis, and the expression of cytokines associated with inflammation and the host defense mechanism. Future *in vitro* studies are required to elucidate the signaling mechanisms underlying the effects of camel milk.

**Acknowledgements**

The present study was supported by a grant from the The Deans of Scientific Affairs, Taif University, Kingdom of Saudi Arabia (grant no. 3281-1-1435).

**References**