Morphological Restructuring of Myocardium During the Early Phase of Experimental Diabetes Mellitus

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ABSTRACT
The purpose of this study was to determine the specific features of the morphological restructuring of the myocardium in the early stage of experimental diabetes mellitus (DM). Experimental type 1 DM rat model was developed by intraperitoneal injection of alloxan solution at a dose of 30 mg per 100 g body mass. After 1 month, 3 mL of blood was drawn by heart puncture and the plasma separated by centrifugation for biochemical analysis. Plasma glucose, insulin, and glycosylated haemoglobin in whole blood were determined. Light microscopy and morphometric studies were conducted of histological slices of the hearts of experimental animals. The investigation of heart morphology showed a statistically significant alteration in chamber wall thickness in the right auricle in rats with alloxan-induced DM. A change in cardiomyocyte diameter in myocardium slices was observed in all chambers of DM rats except for the left ventricle. Average cardiomyocyte diameter in rats with experimental DM increased by 26.6% and 15.5% in the right auricle and right ventricle, respectively, while average cardiomyocyte diameter in the left auricle decreased by 20.8%. Histological investigation of the heart following alloxan injection demonstrated, under the epicardium, distended vessels of the venous collecting microcirculatory system. Aggregation and agglutination of red blood cells and endothelial cell destruction were found in some vessels. In the early stage of DM development, structural alterations in the microcirculatory channels and myocardocytes can be observed in the heart. These structural alterations were most evident in the right chambers of the heart. Anat Rec, 298:396–407, 2015. © 2014 Wiley Periodicals, Inc.

Key words: alloxan; experimental diabetes; early stage; right auricle

INTRODUCTION
While type 2 diabetes mellitus (DM) is the most widespread form of the disease, type 1 DM, found in only 5%–10% of all diabetic cases, is no less significant, both scientifically and clinically, particularly due to the high prevalence of the development of complications in the course of disease progression (Balabolkin et al., 1999;...
Cardiovascular disease and complications are also more prevalent among patients with type 1 or type 2 diabetes compared to patients without diabetes (Krolewski et al., 1987; Laing et al., 2003; Norgaard et al., 1990; Unger and Foster, 1988; Wilson et al., 1998). For instance, type 1 diabetes is associated with at least a 10-fold increase in cardiovascular disease as compared with an age-matched nondiabetic population (Dorman et al., 1984; Laing et al., 2003). Consequently, a considerable number of both experimental (Fein et al., 1980; Joffe et al., 1999; Hoit et al., 1999) and clinical (Drews et al., 2004; Friedman et al., 1982; Regan et al., 1977; Rubler et al., 1972) studies have been devoted to the investigation of cardiovascular complications associated with diabetes.

A strong association between hyperglycemia and cardiovascular disease has been suggested by some (Lehto et al., 1999) but not all (Lloyd et al., 1996; Orchard et al., 2003) researchers. The pattern of development and pathogenesis of these disturbances, however, remain insufficiently studied (Yu et al., 2007). Currently, it is believed that in the pathogenesis of chronic diabetic complications, including cardiovascular complications, the activation of peroxidation processes and a decrease in NADH oxidase activity play critical roles (Baynes and Thorpe 1999; Fiordaliso et al., 2004; Ghosh et al., 2004a, 2004b; Rajagopalan et al., 1996). These alterations lead to, in particular, the development of diabetic cardiomyopathy, whereby cardiomyocyte apoptosis is combined with myocardial hypertrophy and increased collagen deposition (Fiordaliso et al., 2001, 2004; Li et al., 2011). Cardiomyocyte hypertrophy and successive myocardial apoptosis and fibrosis are structural features of diabetic cardiomyopathy that lead to not only anatomic reorganization of the heart primarily via alteration of heart chamber dimensions but also functional disturbances in myocardial contractility in the form of systolic and/or diastolic dysfunctions (Feng et al., 2008).

It is of interest that several studies have reported that in type 1 DM, restructuring occurs only in the left heart chambers due to an increase in the wall thickness of the left ventricle (LV), caused by microcirculation disturbances (Hoit et al., 1999; Karamitsos et al., 2007; Yu et al., 2007). In contrast, several studies have reported that restructuring of the myocardium also takes place on the right side of the heart in type 1 DM (Karamitsos et al., 2007; Kosmala et al., 2007). It is important to note that disturbances of the right heart chambers in patients with DM, in the presence of cardiac insufficiency, pulmonary hypertension or previous myocardial infarction, greatly affect quality of life, and survival prognosis (Karamitsos et al., 2007; Marcu et al., 2006).

Currently, research investigating specific features of the histomorphological restructuring of the myocardium, specifically of the right cardiac chambers, in type 1 DM, remain few (Drews et al., 2004; Kosmala et al., 2007; Wang et al., 2007; Ze-Zhou and Jing, 2008), prompting the present investigation. Few reports have assessed right ventricular (RV) dysfunction or structural abnormality in diabetic cardiomyopathy. For instance, a report in a streptozotocin (STZ)-induced diabetic rat model combined with hypertension caused by renal clipping showed that histopathological findings of myocardial damage were present in both ventricles, but more predominant in the RV wall than in the LV wall (Fein et al., 1989). In terms of the responsible mechanism, the authors speculated lung congestion due to LV failure, which most likely caused RV overload and myocardial damage (Fein et al., 1989). In contrast, in a diabetic model without hypertension, LV ejection fraction (LVEF) was significantly decreased, although LV failure was not severe, indicating that RV remodeling is unlikely to be brought on by backward failure through LV failure (Nemoto et al., 2006).

The majority of studies describe the development of diabetic cardiomyopathy in the later stages of diabetes, generally between the 8th and 12th week after induction of diabetes (Akula et al., 2003). In addition, many studies have aimed to reveal changes in the left chambers of the heart. In contrast, the aim of the present study was to determine the specific features of morphological restructuring of the right chambers of the heart in the early stage of experimental DM.

**MATERIALS AND METHODS**

**Experimental Model of Type 1 DM**

The experimental type 1 DM rat model was modeled by intraperitoneal (i.p) injection of alloxan solution at a dose of 300 mg/kg body mass, in accordance with a modified version of a standard diabetes animal model in outbred male rats (Danilova and Gette, 2009; Loreto and Elina, 2009).

Alloxan produces reactive oxygen species (ROS) with the DNA of the pancreatic islets being one of the targets of ROS (Loreto and Elina, 2009; Malaisse, 1982). In addition, alloxan has a high affinity to SH-containing cellular compounds and, as a result, reduces glutathione content. Furthermore, alloxan inhibits glucokinase, a SH-containing protein essential for insulin secretion induced by glucose (Szkudelski, 2001). Alloxan also has a lower direct cytotoxic effect compared with STZ and alloxan models of diabetes enable more accurate timing of selected metabolic events and their pathophysiologic consequences (Like and Rossini, 1976).

**Animal Preparation and Anesthesia**

The animals used in the study were quarantined in the vivarium of the Institute of Immunology and Physiology of the Ural Division of RAS (Ekaterinburg, Russia). The animals showed no symptoms of any disease. All animals were kept under equal conditions (12 hr light/12 hr dark), and were fed according to the customary schedule with free access to water.

All animal experimental procedures were approved by the Institute of Animal Care and Use Committee at the Institute of Immunology and Physiology of the Ural Division of RAS (Ekaterinburg, Russia) and performed in accordance with the principles formulated at the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, France, 18 March 1986), APs’s Guiding Principles in the Care and Use of Vertebrate Animals.
Animals in Research and Training and in accordance with the Laboratory Practice Regulations of RF (Institute of Laboratory Animal Research, 1996; Ministry of Public Health Order no. 267 from 19 June 2003; Flecknell, 1987; Swindle and Adams, 1988).

Animal Protocol

The experiments were carried out on 20 outbred male rats. The animals were of the same age, received from the rat farm at the same time, and formed the test and the control groups. The animals were 16 weeks old.

The test group (A), consisted of 10 rats with an average body weight of 210 g. These rats received an i.p injection of alloxan, at a dose of 300 mg/kg. For the control of disease progression, plasma glucose was determined at days 3, 7, 17, and 30. The blood was collected from the tail vein.

The control group, Group B consisted of 10 rats with an average body weight of 220 g. These rats received saline injections. After 1 month, the animals from group B were taken out of the experiment and euthanized with saline injections. After 1 month, the animals from group A were taken out of the experiment and euthanized with saline injections. The animals were 16 weeks old.

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Preparation of Samples for Histological and Morphological Studies

Under general ether anesthesia, the thorax cavity was opened by midline thoracotomy. The hearts of rats were arrested in the diastole phase with 2% lidocaine, excited and perfuse-fixed on a Langendorf apparatus under constant pressure with 4% paraformaldehyde in phosphate-buffered saline (PBS). The perfusion pressure was 100 mmHg (Koenig et al., 1945).

The hearts were stored in 4% PBS for 24 hr at +4°C temperature (RT). The standard dehydration procedure was performed (Farmilo and Stead, 2009). The hearts were then longitudinally cut in half through the sagittal plane to determine the left and right chambers of the atrium and the left and right chambers of the ventricle.

The heart tissue was processed and embedded in paraffin wax using the autoprocessor Leica EG 1160 (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) according to the manufacturer’s instructions. Serial 3–5 micron (μm) thick sections were stained with hematoxylin and eosin (H&E) to investigate cellular and myocardial structural changes (Bancroft and Stevens, 1996; Lillie, 1977; Figs. 1–5).

In addition, the hematoxylin-basic fuchsin picric acid (HBFP or Van Gieson’s) staining method was used for detection of myocardial lesions and defects of vessel basement membrane, as previously described (Serov et al., 1976; Prento, 1993; Fig. 6).

For improved visualization of structural changes in the myocardium (i.e., degree of cardiomyocyte and connective tissue stroma destruction), the triphenyl tetrazolium chloride (TTC) staining method employed by Lie et al., was conducted (Figs. 7–10; Lie et al., 1975). This enzymatic staining technique takes advantage of inability of ischemic myocardium (i.e., dehydrogenase-deficient cells) to reduce TTC, resulting in a dark purplish-brown precipitate in ischemic cells.

Heart chamber wall thickness was determined as the length of the perpendicular to the longitudinal axis of the wall (an average of 10 measurements was taken).

The determination of the average diameter of the cardiomyocytes in each section of the heart was carried out on transverse sections of 20 fields at a view of 100× magnification regirding by 1 mm² with the help of the software «Morphology» 5.0 (Video Test, Russia).
Photomicrographs were obtained with the 5× objective for measuring the wall thickness of the heart chambers and 100× for measuring the diameter of cardiomyocytes with the help of the software «Morphology» 5.0 (Video Test, Russia).

Statistical Analysis

Results are expressed as mean ± standard deviation (SD). Data were analyzed using the nonparametric Mann-Whitney test.

RESULTS

The biochemical analysis, 1 month after alloxan injection, revealed that the rats had successfully developed experimental DM. The plasma glucose level in the test animals was 27.8 ± 3.5 mmol/L and 6.0 ± 0.2 mmol/L in the control group (Table 1). Plasma insulin concentrations were 0.45 ± 0.09 mg/L, 62.8% lower than control values of 1.21 ± 0.2 mg/L (P < 0.05), while the glycosylated haemoglobin level increased to 9.6 ± 0.3%, 88.2% higher than the value of 5.1 ± 0.2% measured in the control group (P < 0.05).

The heart morphology investigation revealed a significant alteration of chamber thickness (Table 2) only in the right auricle in rats with alloxan-induced diabetes with an increase of 76.2% compared with the control group (P < 0.05).

A change in cardiomyocyte diameter in myocardium slices was observed in all heart segments except for the left ventricle (Table 3). The average cardiomyocyte diameter in rats with diabetes significantly increased by 28.6% (P < 0.05) and 15.5% (P < 0.05) in the right auricle and right ventricle, respectively, while average cardiomyocyte diameter decreased by 20.8% (P < 0.05) in the left auricle.

The results of histological investigation of the heart of intact animals (group B) are presented in Figs. 1–6. Histological investigation of the heart after alloxan injection demonstrated distended vessels of the venous collecting microcirculatory system under the epicardium (Figs. 1, 2). These investigations also revealed marginal adhesion of red blood cells (RBCs) on the endothelium with (Fig. 2B) and without (Fig. 2D) aggregation. Focal destruction of the basal membrane was observed in some vessels (Fig. 3). In addition to structural disturbances of the microcirculation, projection of the mesothelium and destruction of epicardial cells was observed.

In perifocal zones, there were cardiomyocytes with signs of myomalacia in the form of disappearance of banding and focal sarcoplasm disintegration (Fig. 2). Nucleus-free cardiomyocytes were also observed. This initial stage of the development of diabetic angiopathy is not generally associated with the replacement of degenerated perivascular cardiomyocytes by connective tissue. With the use of TTC staining in the current study, however, interstitial edema and signs of ischemia were observed in test animals while intact animals showed no histological changes (Figs. 4, 5).

In the myocardium of right chambers of the heart, the interstitial edema and the focal destruction of cardiomyocytes formed karyolysis and karyopyknosis of nuclei with signs of plasmorrhexitis observed (Fig. 6).

### Additional Table

**TABLE 1. The plasma glucose levels in the course of experimental DM**

<table>
<thead>
<tr>
<th>Days of the experiment</th>
<th>Control</th>
<th>Alloxan diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 day</td>
<td>5.9 ± 0.25</td>
<td>20.4 ± 3.7*</td>
</tr>
<tr>
<td>7 day</td>
<td>5.85 ± 0.25</td>
<td>26.5 ± 5.3*</td>
</tr>
<tr>
<td>14 day</td>
<td>6.1 ± 0.1</td>
<td>27.0 ± 4.2*</td>
</tr>
<tr>
<td>30 day</td>
<td>6.0 ± 0.2</td>
<td>27.8 ± 3.5*</td>
</tr>
</tbody>
</table>

Note: Statistically significant difference in values in case of paired comparison: *P < 0.05 in comparison with control animals.

**TABLE 2. Wall thickness of heart chambers in the animal groups (µm)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Right</th>
<th>Left</th>
<th>Right</th>
<th>Left</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>443.39±52.35</td>
<td>1007.01±283.24</td>
<td>1138.24±58.70</td>
<td>5002.94±121.82</td>
</tr>
<tr>
<td>Alloxan diabetes</td>
<td>781.41±259.21*</td>
<td>1067.64±162.2</td>
<td>1218.47±150.00</td>
<td>4408.21±676.23</td>
</tr>
</tbody>
</table>

Note: Statistically significant difference in values in case of paired comparison: *P < 0.05 in comparison with control animals.

**TABLE 3. Cardiomyocyte diameter in different heart chambers in the animal groups (µm)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Right</th>
<th>Left</th>
<th>Right</th>
<th>Left</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.258±0.27</td>
<td>13.631±0.72</td>
<td>12.590±0.24</td>
<td>13.356±0.37</td>
</tr>
<tr>
<td>Alloxan diabetes</td>
<td>12.983±0.35*</td>
<td>10.798±0.4*</td>
<td>14.539±0.25*</td>
<td>12.865±0.3</td>
</tr>
</tbody>
</table>

Note: Statistically significant difference in values in case of paired comparison: *P < 0.05 in comparison with control animals.
Thus, it is clear that at early stages of diabetes, diabetic cardiomyopathy occurs primarily in the right chambers of the heart. This manifests itself in the disturbance of endomysium microcirculation (aggregation and agglutination of RBCs), the consequence of which is the development of interstitial edema, tissue hypoxia, and the focal destruction of cardiomyocytes (Figs. 1, 2).

**DISCUSSION**

It is currently widely accepted that pancreatic islet β-cell death occurs in both type 1 and type 2 DM, resulting in absolute or relative insulin deficiency (Donath and Halban, 2004). Consequently, STZ- or alloxan-induced diabetic animal models are considered excellent animal model of type 1 DM. Nevertheless, there are extensive limitations of these diabetic animal models (Donath and Halban, 2004). While a single injection of alloxan or STZ does not usually lead to an autoimmune reaction as observed in type 1 diabetes, these diabetic animal models still lack a significant resistance to increased insulin action as seen in type 2 diabetes (Dyntar et al., 2006).

Regardless, metabolic changes typically observed in type 1 and 2 diabetic patients as characterized by hyperglycemia and increased circulation of free fatty acids (FFA), are induced in both these diabetic animal models. Consequently, in these diabetic animal models, the heart is eventually exposed to the major compounds of a diabetic milieu (Dyntar et al., 2006).

In the current study, the alloxan-induced DM in rats was confirmed by a significantly increased plasma glucose concentration of $27.8 \pm 3.5$ mmol/L in the diabetic group compared with $6.0 \pm 0.2$ mmol/L in the control group when measured 24 hr after the final alloxan injection.

A significant increase in cardiomyocyte diameter in the right chambers of the heart and a significant decrease in cardiomyocyte diameter in the left auricle in test animals ($P < 0.05$) was observed. Cardiomyocyte diameter in the LV of test animals was not significantly different (Table 3). Current studies report various results in terms of changes in cardiomyocyte size in a diabetic milieu. The results of the present study are in accordance with the results of several researchers (Dyntar et al., 2006; Grimm et al., 2002; Nunoda et al., 2002).
1985). In contrast, other studies have reported that cardiomyocyte size in animal experimental diabetes models either altogether change (Kita et al., 1991), or there are variations of only cardiomyocyte length without a change in cardiomyocyte transverse diameter (Cagalinec et al., 2013). Moreover, the results of the present study directly contradict the results of other researchers (Nemoto et al., 2006; Stilli et al., 2007), who observed a significant decrease in cardiomyocyte transverse diameter of cardiomyocytes of both the RV and LV of diabetic hearts. A possible explanation for the ambiguity between the results of previous studies and those of the present study is a difference in the recording time of cardiomyocyte length following the induction of diabetes. For example, while Nemoto et al. (2006) reported cardiovascular morphological changes 18 weeks following STZ-induced diabetes, coinciding with systolic myocardial dysfunction and hypertrophy of the heart ventricles, indications of LV dysfunction have previously been reported in rats 4–6 weeks following the induction of experimental diabetes (Akula et al., 2003; Mihm et al., 2001; Yu et al., 2007). These results contradict the results of a study conducted by Stilli et al. (2007). It is possible that the inconsistencies of reported data in terms of cardiomyocyte length and the progression of diabetes may be due to the employment of simple geometric approximations by previous researchers, which may inherently neglect the complexity of cardiac myocyte shape and length.

The cardiomyocyte hypertrophy observed in the right chambers of diabetic hearts in the current study resulted in a significant increase in right atrium size (Table 2) in the absence of changes in the size of the heart’s left chamber. These results are in accordance with the results of a study by Shioi (2003) yet are in contrast with previous studies in rats (Joffe et al., 1999; Hoit et al., 1999).

The action of the endogenous renin-angiotensin system (RAS) in the heart may be a further explanation for the hypertrophy observed in the right chambers of the hearts of experimental diabetic animals. Cardiac hypertrophy begins as an adaptive response of the heart to...
Fig. 3. Micrograph of section of Van Gieson's st stained myocardium. Section from Group A (hyperglycemic) right ventricular myocardium exhibiting (a) aggregation and agglutination of red blood cells and (b) focal destruction of the basal membranes. Bar = 50 μm.

Fig. 4. Micrographs of right atrial myocardial sections stained using the TTC method. (A) Group B (control) myocardium. (B) Group A (hyperglycemic) myocardium with extensive interstitial edema. Bar = 100 μm.
hemodynamic overload or neurohumoral factors (Yamazaki et al., 1998). In addition, it is well recognized that angiotensin II (Ang-II) and norepinephrine activation of independent signaling pathways are major mechanisms resulting in observed right chamber hypertrophy (Yamazaki and Yazaki, 2000). Indirect evidence supports a role of RAS in the initiation of cell growth in smooth muscle and myocardial cells (Katoh et al., 1989) and inhibition of RAS has been shown to prevent LV hypertrophy in rats with pressure overload (Kromer and Riegger, 1988; Sen, 1983) and to promote the regression of chronic pressure overload hypertrophy in humans (Devereaux et al., 1987). In addition, it has been reported that diabetic cardiomyopathy may be viewed as an Ang-II-dependent process in which the Ang-II peptide plays a critical role in myocyte death and hypertrophy (Fiordaliso et al., 2000).

Angiotensin-converting enzyme (ACE), a membrane-bound enzyme, is widely distributed in cardiac vascular endothelial cells (Balcells et al., 1997). It has previously been shown that hypoxia has various effects on the myocardium of the right and left chambers of the heart, and that while the myocardium of the RV responds to hypoxia with an increase of local activity and increased expression of ACE, the myocardium of the LV responds to hypoxia with a decrease in ACE production (Morrell et al., 1997). Therefore, the action of endogenous ACE in response to hemodynamic overload and hypoxia in the early stages of diabetes may influence the development of hypertrophy observed in the right chambers of experimental diabetic hearts.

Finally, during the prolonged course of diabetes (the stage that the majority of clinical investigations are conducted), the rennin-angiotensin system and the endogenous chymases are activated (Li et al., 2002) resulting in an increase in general peripheral vascular resistance and blood arterial pressure, thus causing LV hypertrophy during the chronic stage of diabetes (Baker et al., 1992; Dedov, 2003).

In the current study, marginal adhesion of RBCs on the endothelium with or without aggregation was observed (Figs. 2 and 5, respectively). The results of the present study are in accordance with the results of several previous studies (Grigoleit et al., 1973; Yalcin et al., 2004; Kim et al., 2006; Sun and Munn, 2006) whereby excessive aggregation of RBCs was observed in diabetic animals. Abnormal rheological dynamics, due to enhanced erythrocyte aggregation, is considered the principal cause of vascular complications in diabetes, as red cell aggregates are unable to pass through capillaries (Grigoleit et al., 1973). Moreover, hemorheological disturbances have been reported to be present in diabetic patients even without clinically detectable microangiopathy (Paisey et al., 1980).

It has recently been suggested that oxidative stress, caused by mitochondrial superoxide overproduction in endothelial cells and hyperglycemia, are the major mechanisms involved in the development of vascular complications in diabetes (Giacco and Brownlee, 2010). Oxidative stress ultimately results in sustained activation of antiangiogenic, proinflammatory pathways even after normalization of glycemia (Giacco and Brownlee, 2010). Hyperglycemia arises early in experimental diabetes. The alloxan-induced rat model of diabetes, investigated in the current study, was characterized by low systemic insulin and marked hyperglycemia (plasma glucose level in test animals was 27.8 ± 3.5 mmol/L). Hyperglycemia results in
activation of the enzyme, aldose reductase. This enzyme converts excess glucose to sorbitol, which in turn is metabolized by sorbitol dehydrogenase to fructose (via the polyol pathway) (Windebank and Feldman, 2001; Singleton et al., 2003). Glucose, particularly sorbitol and fructose, reacts nonenzymatically with proteins, lipids and nucleic acids to produce advanced glycation end products (AGEs), which in turn induce generation of ROS (Singh et al., 2001). Endothelial ROS formation is further increased in hyperglycemic environments by peroxidation of abundant glucose and LDLs and by dysregulation of transition metals that serve as catalysts for auto-oxidation (Cameron et al., 2001). These mechanisms appear to be particularly important for small arterioles, where failure of vasodilation may result in direct tissue ischemia (Singleton et al., 2003). It is possible that the changes in the microcirculatory system observed in the current study, such as distended vessels of the venous collecting microcirculatory system, were a result of the diabetic-induced hyperglycemic environment. In accordance with these findings, it has been shown that hyperglycemia can stimulate apoptosis of microvascular cells through a mechanism not involving other cell types (i.e., via activation of FOXO1) thus further contributing to the development of the microvascular complications associated with diabetes (Behl et al., 2009).

Fig. 6. Micrographs of haematoxylin-eosin stained sections of myocardium. Sections from (A and C) Group B (control) and (B and D) Group A (hyperglycemic) hearts. (A) Left atrium. (B) Left atrium with (a) intact cardiomyocytes, and (b) cardiomyocytes with focal destruction in the form of karyolysis and karyopyknosis. Note marked interstitial edema. (C) Left ventricle with normal myocardium and vasculature. (D) Left ventricle with interstitial edema. Bar = 10 μm.
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LITERATURE CITED


