Decreased Number and Impaired Angiogenic Function of Endothelial Progenitor Cells in Patients With Chronic Renal Failure

Jin-Ho Choi, Koung Li Kim, Wooseong Huh, Beom Kim, Jonghoe Byun, Wonhee Suh, Jidong Sung, Eun-Seok Jeon, Ha-Young Oh, Duk-Kyung Kim

Objective—Increased risk of cardiovascular disease in patients with chronic renal failure (CRF) has been explained by accelerated atherosclerosis and impaired angiogenesis, in which endothelial progenitor cells (EPCs) may play key roles. We hypothesized that altered EPC biology may contribute to the pathophysiology of CRF.

Methods and Results—EPCs were isolated from CRF patients on maintenance hemodialysis (n=44) and from a normal control group (n=30). CRF patients showed markedly decreased numbers of EPC (44.6%) and colonies (75.3%) when compared with the controls (P<0.001). These findings were corroborated by 30.5% decrease in EPC migratory function in response to vascular endothelial growth factor (VEGF) (P=0.040) and 48.8% decrease in EPC incorporation into human umbilical vein endothelial cells (HUVEC) (P<0.001). In addition, Framingham’s risk factor score of both CRF (r = -0.461, P = 0.010) and normal group (r = -0.367, P = 0.016) significantly correlated with the numbers of EPC. Indeed, the number of circulating EPC was significantly lower in CRF patients than in normal group under the same burden of risk factors (P<0.001). A significant correlation was also observed between dialysis dose (Kt/V) and EPC incorporation into HUVEC (r = 0.427, P = 0.004).

Conclusions—EPC biology, which is critical for neovascularization and the maintenance of vascular function, is altered in CRF. Our data strongly suggest that dysfunction of circulating EPC has a role in the progression of cardiovascular disease in patients with CRF. (Arterioscler Thromb Vasc Biol. 2004;24:1246-1252.)

Key Words: endothelial progenitor cell ■ risk factors ■ vascular biology ■ renal physiology

The lifespan of patients with chronic renal failure (CRF) is reduced, and coronary artery disease is the most important cause of morbidity and mortality in these patients.1,2 Even the results of therapeutic strategies such as percutaneous coronary intervention and bypass surgery have shown poor procedural success rates and dismal long-term event-free survival in CRF patients.3,4 Most of the increased cardiovascular morbidity and mortality in CRF has been accounted for by the rapid progression of atherosclerosis, which is clinically shown to be accelerated in CRF.5,6 Experimental studies have also shown that even mild renal dysfunction causes a dramatic acceleration of atherosclerosis.7 Angiogenesis, which is an essential compensation for myocardial ischemia, is also impaired in CRF.8 However the mechanism underlying the acceleration of atherosclerosis and impaired angiogenesis by CRF has not been examined closely. Although the phenomenon has been partially explained by the higher prevalence of established risk factors in CRF, such as hypertension, abnormal carbohydrate metabolism, and increased low density lipoprotein (LDL) cholesterol, the extent and severity of cardiovascular disease is clearly disproportionately high relative to the underlying risk factor profile.9,10 Recent studies have identified that normal adults have a small amount of circulating endothelial progenitor cell (EPC) in the peripheral blood. In response to cytokine stimulation and ischemic insult, these cells are mobilized from bone marrow, home to the ischemic tissue, and contribute to neovascularization and angiogenesis.11-14 Moreover, EPC is regarded to have a key role in the maintenance of vascular integrity and to act as “repair” cells in response to the endothelial injury.15,16 which has been regarded as an initial step in atherosclerosis and a result of the actions of various cardiovascular risk factors.17 Current data suggest that decrease in circulating EPC contributes not only to impaired angiogenesis but also to the progression of atherosclerosis,18 and patients at risk for coronary artery disease have a decreased number of circulating EPC with impaired activity.19-22 Therefore, we reasoned that EPC, which is critical for neovascularization and the maintenance of vascular integrity,
might be numerically or functionally impaired in CRF. This would contribute to the accelerated atherosclerosis and impairment of angiogenesis, which may account for the increased cardiovascular risk and poor clinical outcomes observed in CRF. We investigated the number and angiogenic function of EPC obtained from patients on maintenance hemodialysis and compared them with the corresponding parameters of healthy volunteers.

Methods

Study Subjects

We studied 44 male CRF patients on maintenance hemodialysis and 30 healthy male volunteers who were older than age 21 years. Chronic stable patients on maintenance hemodialysis were selected to exclude potential factors that may affect biology of circulating EPC in the setting of recent aggravation of renal function. Patients with clinical evidence of symptomatic coronary artery disease, peripheral vascular disease, cerebrovascular disease, or carotid artery stenosis were excluded. Subjects with any condition, such as neoplasm, wounds, or significant retinopathy, which might involve neovascularization, were also excluded.14 Medications, including statins, glucose-controlling drugs, and antihypertensive drugs, were continued. No new medications, including vitamins, were used for at least 2 weeks before the study. Laboratory data of each subject were obtained within 2 weeks from the date of blood sampling. All enrolled subjects underwent a detailed assessment of cardiovascular risk after signing an informed consent document approved by the Institutional Review Board of Samsung Medical Center.

Isolation of EPC

Peripheral blood (30 mL) was drawn by venipuncture using a heparin-coated syringe. Samples of CRF patients were drawn just before the beginning of hemodialysis to exclude any possible influence of dialyzer on the cell. Mononuclear cells were isolated by density gradient method using Ficoll-Paque Plus (Amersham, Buckinghamshire, UK), and resuspended in EGM-2 MV Singlequot medium (Cambrex, East Rutherford, NJ), which contains multiple growth factors including human vascular endothelial growth factor (VEGF) A, human fibroblast growth factor-2, human endothelial growth factor, insulin-like growth factor-1, and ascorbic acid. Cells were plated on 6-well plates (Becton Dickinson, Franklin Lakes, NJ) coated with 2% gelatin (Sigma, St. Louis, Mo). The initial seeding density was standardized at 4 \times 10^4 cells per well. After 3 days of culture, nonadherent cells were removed and the media were changed. The culture was maintained through day 7. After the numbers of EPC and EPC colony were counted, cells were assayed or harvested for further study.

EPC Characterization

Endothelial cell (EC) phenotype of EPC was investigated at day 7. Adherent cells were incubated with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)-labeled acetylated LDL (Molecular Probes, Eugene, Ore) at 37°C for 3 hours, and then with fluorescein-isothiocyanate-conjugated Ulex europaeus agglutinin (UEA)-1 lectin (10 \mu g/mL, Sigma) for 4 hours. Samples were examined with an inverted fluorescence microscope (Zeiss) and only cells exhibiting double fluorescence were identified as EPC.21,23 Cultured human umbilical vein endothelial cells (HUVEC) and NIH 3T3 cells were stained simultaneously and served as the positive and negative controls, respectively. To confirm endothelial cell phenotype of EPC, cells were also simultaneously stained with primary antibodies against the VEGF receptor KDR (Sigma) and von Willebrand factor (vWF) (BD Pharmingen, San Diego, Calif). Fluorescein-isothiocyanate and rhodamine-linked secondary monoclonal antibodies were used.

To confirm that the EPCs of our study population were capable of intracellular nitric oxide (NO) synthesis, we examined the EPCs of normal subjects with a membrane NO-specific fluorescence indicator, diaminofluorescein-2 diacetate (DAF-2 DA; Daichi, Japan). EPCs were gently washed twice with calcium-free phosphate-buffered saline (PBS) and bathed in Krebs-Henseleit buffer containing L-arginine (1 mmol/L) and DAF-2 DA (10 \mu mol/L) for 15 minutes. The cells were then incubated at 37°C for an additional 15 minutes, and 50 ng/mL of recombinant human VEGF_{165} (R&D Systems, Minneapolis, Minn) was added to the wells. The samples were then examined with fluorescence microscopy.

Cell surface antigens were investigated with fluorescence-activated cell sorter (FACS) analysis.11,21 Cultured cells were detached by brief incubation with trypsin/1 mmol/L EDTA followed by forceful pipetting, and cells (1 \times 10^6 cells each) were used for FACS analysis. Antibodies to the VEGF receptor KDR (Sigma), VWF (BD Pharmingen), and VE-cadherin (BD Pharmingen) were used as primary antibodies. Peripheral blood mononuclear cells served as controls.

EPC Count

The numbers of EPC and EPC colony were determined by counting 12 random high-power (×100) microscope fields per subject and were expressed as cells or colonies per mm^2. Spindle-shaped cells and colonies consisting of multiple thin, flat cells emanating from a central cluster of rounded cells were counted.

EPC Migration Assay

EPC migratory function, which is essential for angiogenesis, was examined using a modified Boyden chamber technique. A 24-well Transwell apparatus (Coster) was used, with each well containing a 6.5-mm polycarbonate membrane with 8-\mu m pores, coated with type I collagen (Sigma). EPCs (4 \times 10^4) were plated on the membrane, and the chamber was immersed in a 24-well plate, which was filled with growth factor-free EBM-2 culture media or EBM-2 with 50 ng/mL of human VEGF_{165}. After incubation for 24 hours, the membrane was washed briefly with PBS and the upper side of membrane was wiped gently with a cotton ball. The membrane was then removed and stained using Giemsa solution. The magnitude of EPC migration was evaluated by counting the migrated cells in 4 random high-power (×100) microscope fields.

Matrigel Tube Formation Assay

A Matrigel tube formation assay was performed to assess the ability of EPC to incorporate into endothelial cell vascular structures, which is believed to be important in new vessel formation.16 Matrigel (Becton Dickinson) was spread onto 4-well chamber slides (Nalge Nunc). EPCs were marked with DiI (Molecular Probes) to distinguish them from HUVEC. DiI-labeled EPC (1 \times 10^5) and HUVEC (4 \times 10^4) were plated together and incubated at 37°C for 24 hours with EGM-2 culture medium. Incorporated cells were counted from 4 random high-power (×100) microscope fields per each subject. The numbers and line lengths of the circle formed by cells were also calculated.

Statistical Analysis

All data are presented as means±SEM. Continuous variables were evaluated by nonparametric Mann–Whitney test or Wilcoxon signed rank test. Categorical variables were evaluated by Fischer exact test. Univariate correlations were made with Spearman correlation coefficient. Multiple linear regression for the analysis of covariance was used to identify predictors of changes in EPC counts in a multivariate setting. SPSS release 11.0 (SPSS Inc) was used, and differences were considered statistically significant at 2-tailed P<0.05.

Results

Patients Characteristics

Clinical data of the study subjects are summarized in the Table. Patients in the CRF group were 4.6 years older than those in the normal group and had a lower body mass index. Total cholesterol and high-density lipoprotein cholesterol
were significantly lower in the CRF group. All CRF patients were treated with erythropoietin, and most of them (97.7\%, n=43) were using antihypertensive medication. Plasma VEGF levels measured with an enzyme-linked immunosor-

**EPC Characterization**

Culture of total peripheral blood mononuclear cell resulted in the emergence of characteristic spindle-shaped EPCs and EPC colonies characterized by a central cluster of rounded cells surrounded by radiating spindle-shaped cells. EPCs (C through E) and EPC colonies (F to H) were shown to uptake acetylated LDL (C, F) and bind UEA-1 (D, G). Merged images show that most cells are dual-positive (E, H).

**EPC Count**

Significantly fewer EPC colonies were formed from mononuclear cell culture of CRF patients compared with normal controls at day 7 (133.7±21.6 versus 542.5±64.1 per 10\(^3\) mm\(^2\); P<0.001) (Figure 2A through 2C). Significantly fewer EPCs were also identified from CRF patients (10.0±1.2×10\(^3\) versus 17.8±2.0×10\(^3\) per 10\(^3\) mm\(^2\); P<0.001) (Figure 2D through 2F). Despite differences in age, body mass index, total cholesterol, high-density lipoprotein cholesterol, hypertension, and usage of statin, which might
affect EPC biology (Table), 15,18,24 the statistically significant differences were maintained after adjusting for these factors (EPC colonies of CRF versus normal; \( P < 0.001 \), EPC of CRF versus normal; \( P = 0.005 \)) (Figure IIA and IIB, available online at http://atvb.ahajournals.org). There was no difference in the numbers of EPCs or EPC colonies between diabetic and nondiabetic CRF patients.

EPC Migration Assay

The migratory function of EPC in response to VEGF, which is believed to be important during neovascularization, 19 was evaluated using a modified Boyden chamber. The basal EPC migratory functions of CRF patients and normal controls were not significantly different \( (P > 0.05) \). After supplementation with 50 ng/mL of VEGF, the migratory function of EPC in both groups was significantly augmented (baseline versus VEGF-stimulated migration, CRF patients; 14.9±3.3 versus 22.4±3.7 per ×100 high-power field; \( P = 0.001 \), normal group; 19.1±3.2 versus 32.9±5.3 per ×100 high-power field; \( P = 0.016 \)) (Figure 3A through 3E). However, the VEGF-induced augmentation of EPC migration was relatively impaired in CRF. VEGF-induced augmentation of EPC migration was reduced by 52.8% in CRF patients relative to that of the normal group (72.5% increase). F. The reduced increase in EPC migration in CRF was statistically significant. \( *P < 0.05 \).

Matrigel Tube Formation Assay

EPC incorporation into the tubular networks formed by HUVEC was evaluated in culture using Matrigel, which is used to evaluate EC differentiation. 25 Significantly fewer EPCs of CRF patients were incorporated into tubules compared with the EPCs from normal controls (8.2±0.5 per mm² versus 16.0±1.1 per mm², \( P < 0.001 \)) (Figure 4A through 4C), independently of clinical factors described \( (P < 0.001) \) (Figure IID, available online at http://atvb.ahajournals.org). Significantly fewer circles and shorter line lengths of the tubules were also identified (Figure IIIA through IID, available online at http://atvb.ahajournals.org).
Framingham risk score.20,26 The EPC number was signifi-
cantly from the total burden of risk factors according to the
disease, the 10-year coronary artery disease risk was esti-
mated from the normal controls (B). EPC incorporation in the CRF group
was reduced by 51.9% (C). *
P
10 3 mm 2 ;

Figure 4. EPC incorporation into EC and stimulation of EC tube
formation is impaired in CRF. EPCs were labeled with Dil and
cocultured with HUVEC in Matrigel-coated 4-well chamber slide
for 24 hours. Fluorescence and light images of identical fields
were merged. Significantly fewer EPCs from CRF patients were
incorporated into the tubules formed by HUVEC (A) than those
of the normal controls (B). EPC incorporation in the CRF group
was reduced by 51.9% (C). *
P
10 3 mm 2 ;

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was reduced by 51.9% (C). *
P
10 3 mm 2 ;

online at http://atvb.ahajournals.org). There was no difference
in EPC incorporation or tubule formation between diabetic
and nondiabetic CRF.

Risk of Coronary Artery Disease and the Level
of EPC
We next investigated the relationship between the level of
circulating EPC and the risk of coronary artery disease, which
is a clinical consequence of accelerated atherosclerosis and
impaired angiogenesis in CRF.5–8 Because none of our study
subjects showed any clinical evidence of coronary artery
disease, the 10-year coronary artery disease risk was esti-
mated from the total burden of risk factors according to the
Framingham risk score.20,26 The EPC number was significan-
tly inversely correlated with the estimated 10-year coro-

nary artery disease risk in CRF patients (r = −0.367, P = 0.016)
and in the normal group (r = −0.461, P = 0.010) (Figure 5A and 5B), suggesting that a lower level of EPC is
associated with a higher risk of coronary artery disease in
CRF as well as in normal renal function group.20

To compare the EPC numbers in both groups under the
same risk burden, the age-adjusted relative risk of each
subject was estimated.26 EPC numbers were also significantly
inversely correlated with the age-adjusted relative risk, both
in CRF patients (r = −0.422, P = 0.005) and in the normal
group (r = −0.399, P = 0.029). The numbers of EPC were
significantly lower in CRF patients after correction for
age-adjusted relative risk (P < 0.001) (Figure 5C). We next
divided the study subjects into a low-risk group (for whom
the 10-year coronary artery disease risk was <2.0-times the
optimal risk profile) and a high-risk group (≥2.0-times). The
number of EPC in CRF patients was significantly lower than
that in the normal group independently of whether they were
in the low-risk or high-risk group (low-risk group; CRF
versus normal; 11.7 ± 1.6 × 10^4 versus 20.3 ± 2.8 × 10^4 per
10^3 mm^2; P = 0.009, high-risk group; 8.2 ± 1.8 × 10^4 versus
15.3 ± 2.8 × 10^4 per 10^3 mm^2; P < 0.001) (Figure 5D). From
these observations, it would appear that the level of EPC is
reduced in CRF irrespective of the risk burden.

Impact of Dialysis Dose on the EPC
Migratory Function
As shown, a lower number of EPC was correlated with a
higher future coronary artery disease risk. We presumed that
the number or function of EPC might also be related to the
dose of dialysis calculated by Kt/V, which is known to be
related to mortality risk in CRF patients.27 Because a Kt/V of
1.3 is regarded as the minimal standard for adequate dialy-
sis,2,27 we compared the EPC incorporation function of
patients receiving a lower dialysis dose (Kt/V < 1.3) with that
of patients receiving a higher dialysis dose (Kt/V ≥ 1.3). The
EPCs of patients receiving lower doses of dialysis showed
significantly impaired incorporation into EC compared with
those of patients receiving higher doses of dialysis.
(5.3±1.1×10³ versus 8.6±0.5×10³ EPC per 10³ mm², P=0.015) (Figure 5E). Furthermore, the dose of dialysis was significantly correlated with the degree of incorporation of EPC into EC (r=0.427, P=0.004) (Figure 5F). This interesting finding suggests that higher doses of dialysis may be related to improved angiogenic function of EPC. However, other parameters such as the number of EPC, the number of EPC colonies, and migratory functions were not correlated with the dose of dialysis.

Discussion

In this study, we demonstrate that the number of circulating EPC is decreased and the angiogenic activity of EPC is impaired in CRF, a disease in which endothelial dysfunction and impaired angiogenesis have been described.8,28 There is growing evidence that bone marrow-derived EPC participates in the repair of endothelial dysfunction.15,16,20 This process can be divided into 3 stages: mobilization from bone marrow, homing into the sites of vascular injury, and incorporation into the endothelium of the injured or newly formed blood vessels.29 In our study, the number of EPC is decreased in CRF despite no difference in plasma VEGF levels. This implies that there may be a fundamental impairment in the production, mobilization, or half-life of EPC in CRF. The exhaustion of a presumed finite supply of EPC by continuous endothelial damage and consumption of EPC for vascular endothelial repair might also contribute to the decreased EPC numbers,18–20

Current data suggest that EPC may be incorporated into damaged endothelium and may work in concert with existing endothelial cells to form blood vessels rather than forming entirely new vessels.18–20,30 It is interesting that not only EPC migration and incorporation into EC was reduced but that EC network formation was also reduced when EC was cocultured with the EPC of CRF patients. These observations suggest that EPC incorporation into damaged endothelium or neovascularization foci may be impaired in CRF, and that the differentiation of normal EC may also be affected when EPC is functionally impaired. These phenomena could contribute to the deterioration in the repair of damaged endothelium or angiogenesis in CRF.8,28

In our study, the number of EPC was much lower in the CRF group than in the normal group, even when both groups had the same burden of risk factors. Furthermore, the number of EPC was inversely correlated with the estimated future coronary artery disease risk in both groups. These findings are consistent with the clinical observation that coronary artery disease risk in CRF is disproportionately high relative to the underlying risk factor profile.9,10 Our study suggests that EPC mobilization and incorporation into damaged endothelium or neovascularization foci may be impaired in CRF, as it is in other traditional risk factors such as aging, diabetes, and hypercholesterolemia.18–22,31

The dose of dialysis is an index of the removal of uremic toxins and is a key determinant of prognosis for end-stage CRF patients.1,27,32,33 Our data show that the dose of dialysis is inversely related to the capacity for EPC incorporation, which is a principal step in tissue vascularization by bone marrow-derived progenitor cells.16 This result may indicate the mechanism by which the greater removal of uremic toxins by higher doses of dialysis can improve the angiogenic function of EPC and the prognosis of CRF patients.

Possible mechanisms for the altered biology of EPC observed in CRF are presented here. Excessive oxidative stress, which is known to be related to increased cardiovascular risk in CRF, may inhibit the differentation of EPC into the mature EC and contribute to the impaired repair of injured vascular endothelium.34,35 Another candidate is the deficient NO production in CRF.36,37 NO synthase, which has an essential role in mobilization of EPC,38 is blocked by endogenous inhibitory actions of guanidine compounds, which are major components of uremic toxins.39,40 Therefore, deficient NO production might lead to decreased mobilization of EPC from bone marrow. Finally, the function of EPC may also be impaired by uremic toxins, similar to the way activation of T and B lymphocyte is impaired in CRF.41 In our study, the level of blood urea nitrogen, which represents an approximate burden of uremic toxin burden, was weakly correlated to the number of EPC (P=0.06, data not shown). However, considering the fact that the blood urea nitrogen varies widely between measurements and is not an accurate measure of uremic toxin burden, the correlation between the level of uremic toxin and the number of EPC may be suggestive and could hardly conclusive.

Recently a new therapeutic strategy involving the administration of autologous EPC to increase neovascularization has generated great interest.23–44 The results of the present study suggest that if such approach is to reach clinical fruition, the function of the transplanted cells must also be considered. The effects of therapeutic angiogenesis using autologous cell transplantation may not be satisfactory in patients with EPC dysfunction, such as diabetic, aged, or those with CRF. However, our study shows that higher dose of dialysis, which may eliminate the cause of EPC dysfunction, is related to better EPC function. If the angiogenic function of EPC can be improved by modification of the pathophysiology of EPC dysfunction, not only the therapeutic outcome of EPC transplantation but also the future coronary artery disease risk may be improved.

In conclusion, our data demonstrate that EPC is numerically and functionally impaired in CRF, and that this is inversely related to the risk factor score of coronary artery disease. The results of our study may account for the acceleration of atherosclerosis, impairment of angiogenesis, and increased coronary artery disease risk observed in CRF patients.

Acknowledgments

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References


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Figure I.

A B C

D E F

G

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Figure II.

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The number of EPC colony was different between CRF and normal group, independently of clinical factors.

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The number of EPC was different between CRF and normal group, independently of clinical factors.

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The number of migrated EPC was different between CRF and normal group, independently of clinical factors.

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The number of incorporated EPC into EC tubules was different between CRF and normal group, independently of clinical factors.
Figure III.

A

B

C circle number

D line length

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Figure Legends for Supplementary Data

**Figure I.** Identification of the endothelial lineage phenotype of cultured EPC. A-C, EPCs and EPC colony showed expression of endothelial lineage markers, vWF (A) and KDR (B). Merged image shows simultaneous expression of these markers (C). D-F, EPCs were both positive for vWF (D) and DAF2-DA, a NO synthesis indicator (E). Merged image shows specific NO synthesis in vWF-positive cells (F). G, FACS analysis of EPC cultured for one week showed that EPCs express markers consistent with endothelial lineage (KDR; 23.9%, vWF; 53.3%, VE-cadherin; 39.1%). PB-MNC (peripheral blood mononuclear cell) served as controls. Asterisk (*) denotes statistical significance (p<0.05).

**Figure II.** The biological difference of EPC between CRF and normal group was sustained independently of clinical factors. The number of EPC colony (p<0.001) (A), the number of EPC (p=0.005) (B), the number of migrated EPC (C), and the number of incorporated EPC into EC tubules (D) were all different between CRF and normal group, and these statistically significant differences were maintained after adjusting for clinical factors. * p<0.05.

**Figure III.** EPC incorporation into EC and stimulation of EC tube formation is impaired in CRF. DiI-labeled EPCs were co-cultured with HUVEC in Matrigel-coated chamber slide for 24 hours.
Fluorescence and light images of identical fields were merged. A-D, Significantly less tubule formation and shorter tubule line lengths were also identified in the CRF group (A) than in the normal group (B). The graphs show a 27.7% decrease in the number of circles (p=0.001) (C) and a 16.6% decrease in the lengths of circle lines (p=0.029) (D). * p<0.05.