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(54) **Title:** METHODS OF DIAGNOSING POST-OPERATIVE COMPLICATIONS USING SERUM NITRIC OXIDE LEVELS

(57) **Abstract:** Disclosed herein are methods of using serum nitric oxide levels to diagnose subjects as having or likely to develop a post-operative complication.



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METHODS OF DIAGNOSING POST-OPERATIVE COMPLICATIONS USING SERUM NITRIC OXIDE LEVELS**[0001] ACKNOWLEDGEMENT OF GOVERNMENT SUPPORT**

[0002] This invention was made with Government support under HL119968, awarded by the National Institutes of Health. The Government has certain rights in the invention.

[0003] BACKGROUND OF THE INVENTION

[0004] Cardiac surgery with cardiopulmonary bypass (CPB) is associated with a systemic inflammatory response, increase in oxidative stress, and upregulation of inflammatory mediators including C-reactive protein (CRP), interleukin 6 (IL-6), and B-type natriuretic peptide (BNP). Increased systemic inflammation is a risk factor for worse post-operative (postop) outcomes including the development of acute kidney injury (AKI) and atrial fibrillation (AF). It has been shown that inflammation induced oxidative stress leads to reduced nitric oxide (NO) bioavailability and concentration in tissues. See Cai, et al. (2002) Circulation 106:2854-2858 (NO measured in left atrial and aortic samples in a porcine model of AF). Unfortunately, measuring NO in tissues require invasive tissue biopsies, which may increase the risk of postop complications, or may only be performed post-mortem.

[0005] Thus, a need exists for methods for screening and monitoring postop complications such as systemic inflammation, and development of postop AF and AKI, which are particularly prevalent in surgical patients having high morbidity and/or high mortality, in subjects without further increasing their risk of postop complications.

[0006] SUMMARY OF THE INVENTION

[0007] In some embodiments, the present invention provides a method of diagnosing a subject as having or likely to develop a postoperative complication after an operation, which comprises measuring the concentration of serum nitric oxide in a first blood sample obtained from the subject before the operation; measuring the concentration of serum nitric oxide in a second blood sample obtained from the subject at about 24 hours after the operation; calculating the percent decrease of serum nitric oxide concentration from the concentration of serum nitric oxide in the first blood sample to the concentration of the serum nitric oxide in the second blood sample; and diagnosing the subject as having or likely to develop the postoperative complication where the percent decrease of the serum nitric oxide concentration is at least about 40%, about 40%, about 40-45%, about 40-50%, about 45-50%, or about 55-60%. In some embodiments, the method further comprises diagnosing the subject as having or likely to develop atrial

fibrillation where the percent decrease of the serum nitric oxide concentration is at least about 40%, about 40%, about 40-45%, about 40-50%, about 45-50%. In some embodiments, the method further comprises diagnosing the subject as having or likely to develop acute kidney injury where the percent decrease of the serum nitric oxide concentration is about 55-60%. In some embodiments, the methods further comprise treating the subject for the postoperative complication where the subject is diagnosed as having the postoperative complication. In some embodiments, the methods exclude treating the subject for the postoperative complication where the subject is diagnosed as not having or not likely to develop the postoperative complication.

[0008] In some embodiments, the present invention provides a method of diagnosing a subject as having or likely to develop a postoperative complication after an operation, which comprises measuring the concentration of serum nitric oxide in a first blood sample obtained from the subject before the operation; measuring the concentration of serum nitric oxide in a second blood sample obtained from the subject at about 24 hours after the operation; calculating the percent decrease of serum nitric oxide concentration from the concentration of serum nitric oxide in the first blood sample to the concentration of the serum nitric oxide in the second blood sample; and diagnosing the subject as having or likely to develop the postoperative complication where the percent decrease of the serum nitric oxide concentration is at least about 28% more, about 28% more, about 28-53% more, about 28-58% more, about 53-58% more, or about 64-67% more than the average percent decrease exhibited by normal control subjects. In some embodiments, the method further comprises diagnosing the subject as having or likely to develop atrial fibrillation where the percent decrease of the serum nitric oxide concentration is at least about 28% more, about 28% more, about 28-53% more, about 28-58% more, about 53-58% more than the average percent decrease exhibited by normal control subjects. In some embodiments, the method further comprises diagnosing the subject as having or likely to develop acute kidney injury where the percent decrease of the serum nitric oxide concentration is about 64-67% more than the average percent decrease exhibited by normal control subjects. In some embodiments, the methods further comprise treating the subject for the postoperative complication where the subject is diagnosed as having or likely to develop the postoperative complication. In some embodiments, the methods exclude treating the subject for the postoperative complication where the subject is diagnosed as not having or not likely to develop the postoperative complication.

[0009] In some embodiments, the present invention provides a method of characterizing a postop blood sample as being from a subject who has or is likely to have a postoperative complication after an operation, said postop blood sample being obtained from the subject about 24 hours after the operation, which comprises measuring the concentration of serum nitric oxide in the postop blood sample and calculating the percent decrease in serum nitric oxide concentration from the concentration of serum nitric oxide in a preop blood sample obtained from the subject before the operation, and characterizing the postop blood sample as being from a subject who has or is likely to develop the postoperative complication where (a) the percent decrease of the serum nitric oxide concentration is at least about 40%, about 40%, about 40-45%, about 40-50%, about 45-50%, or about 55-60%, or (b) the percent decrease of the serum nitric oxide concentration is at least about 28% more, about 28% more, about 28-53% more, about 28-58% more, about 53-58% more, or about 64-67% more than the average percent decrease exhibited by normal control subjects. In some embodiments, the method further comprises categorizing the postop blood sample as being from a subject who has or is likely to develop atrial fibrillation where the percent decrease of the serum nitric oxide concentration is at least about 40%, about 40%, about 40-45%, about 40-50%, about 45-50%. In some embodiments, the method further comprises characterizing the postop blood sample as being from a subject who has or is likely to develop acute kidney injury where the percent decrease of the serum nitric oxide concentration is about 55-60%. In some embodiments, the method further comprises characterizing the postop blood sample as being from a subject who has or is likely to develop atrial fibrillation where the percent decrease of the serum nitric oxide concentration is at least about 28% more, about 28% more, about 28-53% more, about 28-58% more, about 53-58% more than the average percent decrease exhibited by normal control subjects. In some embodiments, the method further comprises characterizing the postop blood sample as being from a subject who has or is likely to develop acute kidney injury where the percent decrease of the serum nitric oxide concentration is about 64-67% more than the average percent decrease exhibited by normal control subjects. In some embodiments, the method further comprises measuring the serum nitric oxide concentration in the preop blood sample.

[0010] In some embodiments of these methods, the blood samples are plasma samples. In some embodiments of these methods, the concentrations of serum nitric oxide in the blood samples are measured using a detectable label added thereto. In some

embodiments of these methods, the operation is cardiac surgery. In some embodiments of these methods, the operation is cardiac surgery with cardiopulmonary bypass.

[0011] Both the foregoing general description and the following detailed description are exemplary and explanatory only and are intended to provide further explanation of the invention as claimed. The accompanying drawings are included to provide a further understanding of the invention and are incorporated in and constitute part of this specification, illustrate several embodiments of the invention, and together with the description serve to explain the principles of the invention.

[0012] DESCRIPTION OF THE DRAWINGS

[0013] This invention is further understood by reference to the drawings wherein:

[0014] Figures 1A-1C show the change in circulating inflammatory markers following cardiac surgery with cardiopulmonary bypass. Figure 1A is a graph showing the postop change in serum nitric oxide (NO) levels. Figure 1B is a graph showing the postop change in B-type natriuretic peptide (BNP). Figure 1C is a graph showing the postop change in C-reactive protein (CRP). Circulating NO dropped significantly at 24 hour postop, which is the time frame when postop AF develops most frequently. Following the drop in NO, BNP, and CRP levels went up, indicating persistent inflammatory activation * = $p < 0.001$.

[0015] Figures 2A-2B show changes in serum NO levels in patients with/without AKI (Figure 2A) or AF (Figure 2B); of note, changes in NO levels during the first 24 hours was clearly evident in patients with AF or AKI, but not in those without.

[0016] Figure 3 is a graph showing the median change in serum NO levels from baseline preop to 24, 48, and 72 hours postop in patients with and without postoperative atrial fibrillation (AF). Patients who developed AF had larger decline in serum NO levels. All subjects, $n=75$.

[0017] Figure 4 is a graph showing the median serum B-type natriuretic peptide (BNP) levels pre- and postop (24hr, 48hr, and 72hr) in patients with and without postoperative atrial fibrillation (AF). Patients with AF had higher BNP levels. All subjects, $n=75$.

[0018] Figures 5A-5C show the median serum NO, B-type Natriuretic Peptide (BNP), and interleukin-6 (IL-6) baseline levels (Preop) as compared to 24, 48, and 72 hours following cardiac surgery with cardiopulmonary bypass (24hr postop, 48hr postop, and 72hr postop). Figure 5A is a graph showing the postop change in serum nitric oxide (NO) levels. Figure 5B is a graph showing the postop change in B-type natriuretic

peptide (BNP). Figure 5C is a graph showing the postop change in interleukin-6 (IL-6). Levels are in μM . * $p < 0.001$, $n = 111$.

[0019] Figure 6 is a graph showing the serum NO % change in subjects with and without AF. All subjects, $n = 111$, Postop AF, $n = 36$ (33%), Without AF, $n = 75$.

[0020] DETAILED DESCRIPTION OF THE INVENTION

[0021] As disclosed herein, inflammation following cardiac surgery with cardiopulmonary bypass (CPB) decreases serum nitric oxide (NO) level, and this reduction in NO bioavailability can be a risk factor for development of postoperative complications including acute kidney injury (AKI) and atrial fibrillation (AF).

[0022] As disclosed herein, there is a decrease in serum NO following cardiac surgery, representing reduced bioavailability likely due to a systemic inflammatory response induced by CPB. This reduction in circulating NO is observed prior to the peaks of B-type natriuretic peptide (BNP) and C-reactive protein (CRP) levels (Figures 1A-1C) and prior to the peaks of BNP and interleukin-6 (IL-6) (Figures 5A-5C). Thus, serum NO levels can be used to detect postoperative systemic inflammation before other inflammatory biomarkers are observed. The experimental data also indicate that while reduced serum NO levels may be caused by initial inflammation resulting from surgery, the serum NO levels might activate feed-forward mechanisms that sustain inflammatory responses, such as those with elevated BNP and CRP levels. The experiments herein show that patients who developed AKI or AF have a larger decrease from baseline in serum NO levels, at 24 hours postop compared to those who did not develop AKI or AF (Figures 2A-2B, Figure 3, and Figure 6). Therefore, serum NO levels may be used to diagnosis, monitor, and/or treat postoperative complications following, for example, cardiac surgery with cardiopulmonary bypass.

[0023] Specifically, as disclosed herein, subjects who developed AKI exhibited about a 55-60% decrease in serum NO at 24 hours postop whereas subjects who did not develop AKI exhibited only about a 33-36% decrease in serum NO at 24 hours postop. Similarly, of the 47, 64, and 75 patient sample sizes, subjects who developed AF exhibited about a 40-45% decrease in serum NO at 24 hours postop, whereas subjects who did not develop AF exhibited only about a 27-33% decrease in serum NO at 24 hours postop. Of the 111 patient sample size, subjects who developed AF exhibited about a 49% decrease in serum NO 24 hours postop and subjects who did not develop AF exhibited only about a 31% decrease in serum NO 24 hours postop.

[0024] Therefore, in some embodiments, the present invention is directed to a method of diagnosing a subject as having or likely to develop a postoperative complication after an operation which comprises diagnosing the subject as having or likely to develop a postoperative complication where the subject's serum NO at 24 hours postop is at least about 40% less than the subject's serum NO prior to the operation. In some embodiments, the present invention is directed to a method of diagnosing a subject as having or likely to develop a postoperative complication after an operation which comprises diagnosing the subject as having or likely to develop a postoperative complication where the subject's serum NO at 24 hours postop is about 40-45% less than the subject's serum NO prior to the operation. In some embodiments, the present invention is directed to a method of diagnosing a subject as having or likely to develop a postoperative complication after an operation which comprises diagnosing the subject as having or likely to develop a postoperative complication where the subject's serum NO at 24 hours postop is about 40-50% less than the subject's serum NO prior to the operation. In some embodiments, the present invention is directed to a method of diagnosing a subject as having or likely to develop a postoperative complication after an operation which comprises diagnosing the subject as having or likely to develop a postoperative complication where the subject's serum NO at 24 hours postop is about 55-60% less than the subject's serum NO prior to the operation. In some embodiments, where the subject's serum NO is about 40-45% less than the subject's serum NO prior to the operation, the subject is diagnosed as having or likely to develop AF as the postoperative complication. In some embodiments, where the subject's serum NO is about 40-50% less than the subject's serum NO prior to the operation, the subject is diagnosed as having or likely to develop AF as the postoperative complication. In some embodiments, where the subject's serum NO is about 55-60% less than the subject's serum NO prior to the operation, the subject is diagnosed as having or likely to develop AKI as the postoperative complication. In some embodiments, the operation is cardiac surgery. In some embodiments, the operation is cardiac surgery with CPB. In some embodiments, the method comprises obtaining a blood sample from the subject and measuring the amount of NO in the sample using a detectable label added thereto. In some embodiments, the method comprises calculating the difference between the amount of serum NO in a first blood sample obtained from the subject before the operation and the amount of serum NO in a second blood sample obtained from the subject about 24 hours after the operation. In some embodiments, the blood sample is a serum sample.

- [0025] As disclosed herein, at 24 hours after an operation, subjects who developed AKI exhibited a negative change in serum NO that was about 64-67% more than that of subjects who did not develop AKI. Similarly, at 24 hours after an operation, subjects who developed AF exhibited a negative change in serum NO that was about 28-53% (in the 47, 64, and 75 patient sample sizes) and about 58% (in the 111 patient sample size) more than that of subjects who did not develop AF.
- [0026] Therefore, in some embodiments, the present invention is directed to a method of diagnosing a subject as having or likely to develop a postoperative complication after an operation which comprises diagnosing the subject as having or likely to develop a postoperative complication where, at 24 hours postop, the subject exhibits a negative change in serum NO that is at least about 28% more than that of a normal control (e.g., the average negative change in serum NO at 24 hours postop from a pool of subjects who did not develop a postoperative complication). In some embodiments, the present invention is directed to a method of diagnosing a subject as having or likely to develop a postoperative complication after an operation which comprises diagnosing the subject as having or likely to develop a postoperative complication where, at 24 hours postop, the subject exhibits a negative change in serum NO that is about 28-53% more or about 28-58% more than that of a normal control. In some embodiments, the present invention is directed to a method of diagnosing a subject as having or likely to develop a postoperative complication after an operation which comprises diagnosing the subject as having or likely to develop a postoperative complication where, at 24 hours postop, the subject exhibits a negative change in serum NO that is about 64-67% more than that of a normal control. In some embodiments, where the subject's serum NO is about 28-53% more or about 28-58% more than that of a normal control, the subject is diagnosed as having or likely to develop AF as the postoperative complication. In some embodiments, where the subject's serum NO is about 64-67% more than that of a normal control, the subject is diagnosed as having or likely to develop AKI as the postoperative complication. In some embodiments, the operation is cardiac surgery. In some embodiments, the operation is cardiac surgery with CPB. In some embodiments, the method comprises obtaining a blood sample from the subject and measuring the amount of NO in the sample using a detectable label added thereto. In some embodiments, the method comprises calculating the difference between the amount of serum NO in a first blood sample obtained from the subject before the operation and the amount of serum NO in a second blood sample obtained from the subject about 24 hours after the operation. In some embodiments, the blood sample is a serum sample.

[0027] In some embodiments, the methods further comprise, after diagnosing the subject as having or likely to develop the postoperative complication, treating the subject for the postoperative complication. For example, if a subject is determined to have a likelihood of developing AF as a postop complication, the subject may be administered one or more pharmacologic agents, such as amiodarone, colchicine, beta-blockers, and the like, which are known or used to prevent or treat AF before AF occurs. Alternatively or in addition to treatment with one or more pharmacologic agents, the subject may be subjected to atrial pacing protocols. If a subject is determined to have a likelihood of developing AKI as a postop complication, the subject may be treated with optimization of fluid status and correction of volume deficits to maintain a mean blood pressure of >65 mmHg and central venous pressure of >8-12 mmHg and/or by reducing dosage or replacing nephrotoxic medications postoperatively to prevent or treat AKI before AKI occurs.

[0028] The following examples are intended to illustrate but not to limit the invention.

[0029] INITIAL STUDIES

[0030] Prospectively enrolled 64 consecutive adult patients undergoing cardiac surgery with CPB were evaluated. Patients with end stage renal disease on hemodialysis, and patients with 1 or more episodes of AF 30 days preoperatively were excluded. Peripheral blood samples were drawn at 4 time points: preoperatively, and postoperatively at 24, 48, and 72 hours. CRP, IL-6, and BNP were measured in blood samples using methods known in the art, and NO levels were measured using a Griess reaction for nitrite/nitrate assay. See Giustarini et al. "Nitrite and Nitrate Measurement by Griess Reagent in Human Plasma: Evaluation of Interferences and Standardization" *Methods in Enzymology*, 2008, Elsevier, Vol. 440, pp. 361-380, which is herein incorporated by reference in its entirety. In brief, 1 ml of plasma was incubated with 10 mg of protamine sulfate for 5 minutes at room temperature. After centrifugation at 10,000 rpm for 10 minutes, 150 μ L of supernatant was mixed with equal volume of modified Griess reagent (Sigma) at room temperature for 5 minutes. Optical density at 540 nm was measured using a Bio-Tek Plate Reader®. Nitrate levels were calculated from a standard curve prepared with known concentrations of sodium nitrate (0-10 μ M). Calibration of the assay was performed using mouse serum and then optimized for human serum testing.

[0031] Postop outcomes included length of hospital stay, AKI defined by the Acute Kidney Injury Network criteria (see Mehta, et al. (2007) *Crit Care* 11:R31 (which is

herein incorporated by reference in its entirety), and AF defined as an irregular rhythm with no discernible P waves lasting greater than 30 seconds.

[0032] A significant reduction in median serum NO levels was observed after cardiac surgery with CPB; from 3.30 μM preoperatively to a nadir of 1.92 μM ($n = 64$, $p < 0.001$) at 24 hours postop. The nadir of postop serum NO preceded the peak of median CRP and BNP (Figure 1). There was a trend toward a larger decrease in serum NO at 24 hours postop in patients who developed AKI ($n = 12$, $\Delta \text{NO} = 1.80 \mu\text{M}$; no AKI: $n = 50$, $\Delta \text{NO} = 1.08 \mu\text{M}$) and AF ($n = 20$, $\Delta \text{NO} = 1.40 \mu\text{M}$; no AF: $n = 44$, $\Delta \text{NO} = 1.09 \mu\text{M}$). Thus, subjects who developed AKI exhibited about a 55% decrease in serum NO 24 hours postop and subjects who did not develop AKI exhibited only about a 33% decrease in serum NO 24 hours postop. Said another way, at 24 hours postop, subjects who developed AKI exhibit a negative change in serum NO that is about 67% more than that of subjects who do not develop AKI. Similarly, subjects who developed AF exhibited about a 42% decrease in serum NO 24 hours postop and subjects who did not develop AF exhibited only about a 33% decrease in serum NO 24 hours postop. In other words, at 24 hours postop, subjects who developed AF exhibit a negative change in serum NO that is about 28% more than that of subjects who do not develop AF.

[0033] This is consistent with results from 47 patients in which a significant reduction in median serum NO levels was observed after cardiac surgery with CPB; from 3.01 μM preoperatively to a nadir of 1.71 μM ($n = 47$, $p < 0.001$) at 24 hours postop. The nadir of postop serum NO preceded the peak of median CRP and BNP. There was a trend toward a larger decrease in serum NO at 24 hours postop in patients who developed AKI ($n = 11$, $\Delta \text{NO} = 1.77 \mu\text{M}$; no AKI: $n = 36$, $\Delta \text{NO} = 1.08 \mu\text{M}$) and AF ($n = 13$, $\Delta \text{NO} = 1.32 \mu\text{M}$; no AF: $n = 34$, $\Delta \text{NO} = 0.86 \mu\text{M}$). Thus, subjects who developed AKI exhibited about a 59% decrease in serum NO 24 hours postop and subjects who did not develop AKI exhibited only about a 36% decrease in serum NO 24 hours postop. Said another way, at 24 hours postop, subjects who developed AKI exhibit a negative change in serum NO that is about 64% more than that of subjects who do not develop AKI. Similarly, subjects who developed AF exhibited about a 44% decrease in serum NO 24 hours postop and subjects who did not develop AF exhibited only about a 29% decrease in serum NO 24 hours postop. In other words, at 24 hours postop, subjects who developed AF exhibit a negative change in serum NO that is about 53% more than that of subjects who do not develop AF.

[0034] FURTHER STUDIES

[0035] In further studies, 75 consecutive adult patients undergoing cardiac surgery with CPB were enrolled and evaluated as above.

[0036] A significant reduction in median serum NO levels was observed after cardiac surgery with CPB; from 3.34 μM preoperatively to a nadir of 1.96 μM ($n = 75$, $p < 0.0001$) at 24 hours post-op. The nadir of postop serum NO preceded the peak of median CRP and BNP (Figures 1 and 4). There was a trend toward a larger decrease in serum NO at 24 hours postop in patients who developed AF ($n = 23$, $\Delta \text{NO} = 1.33 \mu\text{M}$; no AF: $n = 52$, $\Delta \text{NO} = 0.91 \mu\text{M}$). Thus, subjects who developed AF exhibited about a 40% decrease in serum NO 24 hours postop and subjects who did not develop AF exhibited only about a 27% decrease in serum NO 24 hours postop. In other words, at 24 hours postop, subjects who developed AF exhibit a negative change in serum NO that is about 46% more than that of subjects who do not develop AF.

[0037] ADDITIONAL STUDIES

[0038] In additional studies, 111 consecutive adult patients undergoing cardiac surgery with CPB were enrolled and evaluated as above.

[0039] A significant reduction in median serum NO levels was observed after cardiac surgery with CPB; from 3.67 μM preoperatively to a nadir of 2.16 μM ($n=111$, $p < 0.0001$) at 24 hours postop. As before, the nadir of postop serum NO preceded the peak of median CRP (Figure 5). There was a trend toward a larger decrease in serum NO at 24 hours postop in patients who developed AF ($n=36$, $\Delta \text{NO}=1.33 \mu\text{M}$; no AF: $n=75$, $\Delta \text{NO}=0.9 \mu\text{M}$). Thus, in this larger cohort, subjects who developed AF exhibited about a 49% decrease in serum NO 24 hours postop and subjects who did not develop AF exhibited only about a 31% decrease in serum NO 24 hours postop. In other words, at 24 hours postop, subjects who developed AF exhibit a negative change in serum NO that is about 58% more than that of subjects who do not develop AF.

[0040] EXEMPLARY EMBODIMENTS

[0041] The following methods are exemplary and are not intended to further limit the scope of the claims.

[0042] Method 1. A method of diagnosing a subject as having or likely to develop a postoperative complication after an operation, which comprises diagnosing the subject as having or likely to develop the postoperative complication where the subject's serum nitric oxide level, at 24 hours after the operation, is at least about 40% less than the subject's serum nitric oxide level prior to the operation.

- [0043] Method 2. A method of diagnosing a subject as having or likely to develop a postoperative complication after an operation, which comprises diagnosing the subject as having or likely to develop the postoperative complication where, at 24 hours after the operation, the subject exhibits a negative change in serum nitric oxide that is at least about 28% more than that of a normal control.
- [0044] Method 3. A method of diagnosing a subject as having or likely to develop a postoperative complication after an operation, which comprises diagnosing the subject as having or likely to develop the postoperative complication where the subject's serum nitric oxide level, at 24 hours after the operation, is about 40-45% less than the subject's serum nitric oxide level prior to the operation.
- [0045] Method 4. A method of diagnosing a subject as having or likely to develop a postoperative complication after an operation, which comprises diagnosing the subject as having or likely to develop the postoperative complication where, at 24 hours postop, the subject exhibits a negative change in serum nitric oxide that is about 28-53% more than that of the normal control.
- [0046] Method 5. A method of diagnosing a subject as having or likely to develop a postoperative complication after an operation, which comprises diagnosing the subject as having or likely to develop a postoperative complication where the subject's serum nitric oxide level, at 24 hours after the operation, is about 55-60% less than the subject's serum nitric oxide level prior to the operation.
- [0047] Method 6. A method of diagnosing a subject as having or likely to develop a postoperative complication after an operation, which comprises diagnosing the subject as having or likely to develop the postoperative complication where, at 24 hours postop, the subject exhibits a negative change in serum nitric oxide that is about 64-67% more than that of the normal control.
- [0048] Method 7A. A method of diagnosing a subject as having or likely to develop atrial fibrillation after an operation, which comprises diagnosing the subject as having or likely to develop atrial fibrillation where, at 24 hours after the operation, the subject exhibits a negative change in serum nitric oxide that is at least about 28% more than that of a normal control.
- [0049] Method 7B. A method of diagnosing a subject as having or likely to develop atrial fibrillation after an operation, which comprises diagnosing the subject as having or likely to develop atrial fibrillation where, at 24 hours postop, the subject exhibits a negative change in serum nitric oxide that is about 28-53% more than that of the normal control.

- [0050] Method 8A. A method of diagnosing a subject as having or likely to develop acute kidney injury after an operation, which comprises diagnosing the subject as having or likely to develop acute kidney injury where the subject's serum nitric oxide level, at 24 hours after the operation, is about 40-45% less than the subject's serum nitric oxide level prior to the operation.
- [0051] Method 8B. A method of diagnosing a subject as having or likely to develop acute kidney injury after an operation, which comprises diagnosing the subject as having or likely to develop acute kidney injury where the subject's serum nitric oxide level, at 24 hours after the operation, is about 55-60% less than the subject's serum nitric oxide level prior to the operation.
- [0052] In some embodiments of any one of the Methods 1-8B above, the operation is cardiac surgery. In some embodiments of any one of the Methods 1-8B above, the operation is cardiac surgery with cardiopulmonary bypass. In some embodiments of any one of the Methods 1-8B above, the method further comprises obtaining a blood sample from the subject and measuring the amount of nitric oxide in the sample using a detectable label added thereto. In some embodiments of any one of the Methods 1-8B above, the method further comprises calculating the difference between the amount of serum nitric oxide in a first blood sample obtained from the subject before the operation and the amount of serum nitric oxide in a second blood sample obtained from the subject about 24 hours after the operation. In some embodiments of any one of the Methods 1-8B above, the blood sample is a serum sample. In some embodiments of any one of the Methods 1-8B above, the method further comprises treating the subject for the postoperative complication where the subject is diagnosed as having the postoperative complication. In some embodiments of any one of the Methods 1-8B above, the subject is not treated for the postoperative complication where the subject is diagnosed as not having or not likely to develop the postoperative complication.
- [0053] All scientific and technical terms used in this application have meanings commonly used in the art unless otherwise specified.
- [0054] As used herein, the term "subject" includes humans and non-human animals. The term "non-human animal" includes all vertebrates, e.g., mammals and non-mammals, such as non-human primates, horses, sheep, dogs, cows, pigs, chickens, and other veterinary subjects and test animals.
- [0055] The use of the singular can include the plural unless specifically stated otherwise. As used in the specification and the appended claims, the singular forms "a", "an", and

“the” can include plural referents unless the context clearly dictates otherwise. The use of “or” can mean “and/or” unless stated otherwise. As used herein, “and/or” means “and” or “or”. For example, “A and/or B” means “A, B, or both A and B” and “A, B, C, and/or D” means “A, B, C, D, or a combination thereof” and said “combination thereof” means any subset of A, B, C, and D, for example, a single member subset (e.g., A or B or C or D), a two-member subset (e.g., A and B; A and C; etc.), or a three-member subset (e.g., A, B, and C; or A, B, and D; etc.), or all four members (e.g., A, B, C, and D).

[0056] To the extent necessary to understand or complete the disclosure of the present invention, all publications, patents, and patent applications mentioned herein are expressly incorporated by reference therein to the same extent as though each were individually so incorporated.

[0057] Having thus described exemplary embodiments of the present invention, it should be noted by those skilled in the art that the within disclosures are exemplary only and that various other alternatives, adaptations, and modifications may be made within the scope of the present invention. Accordingly, the present invention is not limited to the specific embodiments as illustrated herein, but is only limited by the following claims.

WHAT IS CLAIMED IS:

1. A method of diagnosing a subject as having or likely to develop a postoperative complication after an operation, which comprises
 - measuring the concentration of serum nitric oxide in a first blood sample obtained from the subject before the operation;
 - measuring the concentration of serum nitric oxide in a second blood sample obtained from the subject at about 24 hours after the operation;
 - calculating the percent decrease of serum nitric oxide concentration from the concentration of serum nitric oxide in the first blood sample to the concentration of the serum nitric oxide in the second blood sample; and
 - diagnosing the subject as having or likely to develop the postoperative complication where the percent decrease of the serum nitric oxide concentration is at least about 40%.
2. The method according to claim 1, wherein the percent decrease is about 40-45% or about 55-60%.
3. The method according to claim 1, wherein the percent decrease is about 40-50% or about 45-50%.
4. A method of diagnosing a subject as having or likely to develop a postoperative complication after an operation, which comprises
 - measuring the concentration of serum nitric oxide in a first blood sample obtained from the subject before the operation;
 - measuring the concentration of serum nitric oxide in a second blood sample obtained from the subject at about 24 hours after the operation;
 - calculating the percent decrease of serum nitric oxide concentration from the concentration of serum nitric oxide in the first blood sample to the concentration of the serum nitric oxide in the second blood sample; and
 - diagnosing the subject as having or likely to develop the postoperative complication where the percent decrease of the serum nitric oxide concentration is at least about 28% more than the average percent decrease exhibited by normal control subjects.
5. The method according to claim 4, wherein the percent decrease is about 28-53% more or about 64-67% more than the average percent decrease exhibited by normal control subjects.

6. The method according to claim 4, wherein the percent decrease is about 28-58% more or about 53-58% more than the average percent decrease exhibited by normal control subjects.
7. The method according to any one of claims 1 to 3, which further comprises diagnosing the subject as having or likely to develop atrial fibrillation where the percent decrease of the serum nitric oxide concentration is about 40-45% or about 40-50%.
8. The method according to any one of claims 4 to 6, which further comprises diagnosing the subject as having or likely to develop atrial fibrillation where the percent decrease of the serum nitric oxide concentration is about 28-53% more or about 28-58% more than the average percent decrease exhibited by normal control subjects.
9. The method according to any one of claims 1 to 3, which further comprises diagnosing the subject as having or likely to develop acute kidney injury where the percent decrease of the serum nitric oxide concentration is about 55-60%.
10. The method according to any one of claims 4 to 6, which further comprises diagnosing the subject as having or likely to develop acute kidney injury where the percent decrease of the serum nitric oxide concentration is about 64-67% more than the average percent decrease exhibited by normal control subjects.
11. The method according to any one of the preceding claims, wherein the first blood sample and the second blood sample are plasma samples.
12. The method according to any one of the preceding claims, wherein the concentrations of serum nitric oxide in the first blood sample and the second blood sample are measured using a detectable label added thereto.
13. The method according to any one of the preceding claims, wherein the operation is cardiac surgery.
14. The method according to any one of the preceding claims, wherein the operation is cardiac surgery with cardiopulmonary bypass.

15. The method according to any one of the preceding claims, which further comprises treating the subject for the postoperative complication where the subject is diagnosed as having or likely to develop the postoperative complication.

16. The method according to any one of the preceding claims, wherein the subject is not treated for the postoperative complication where the subject is diagnosed as not having or not likely to develop the postoperative complication.

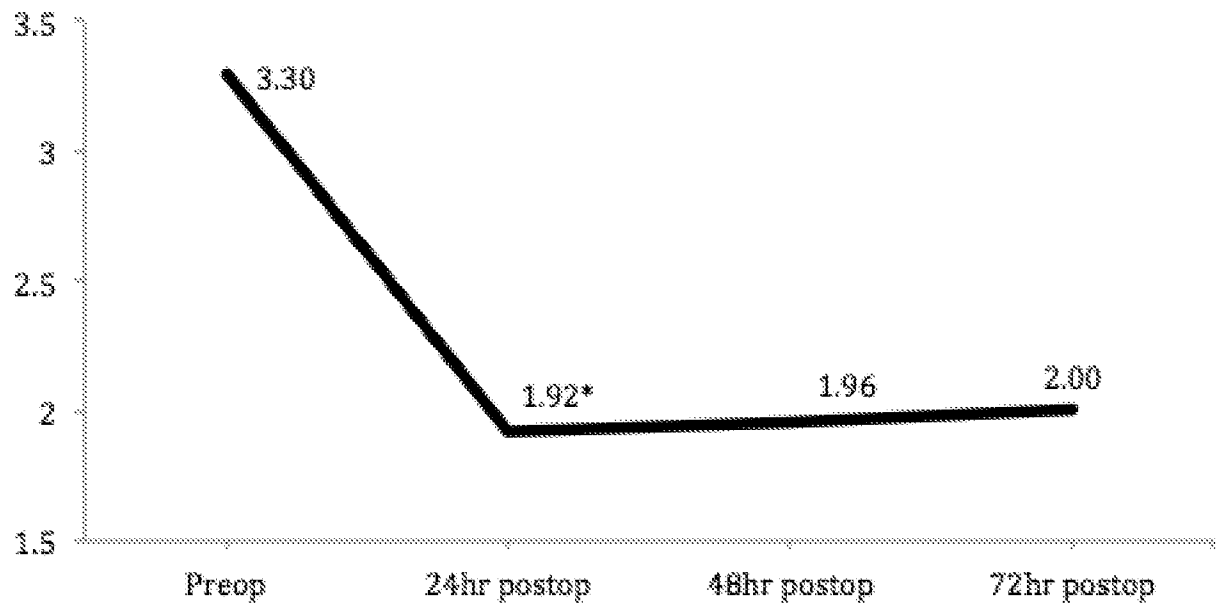


Figure 1A

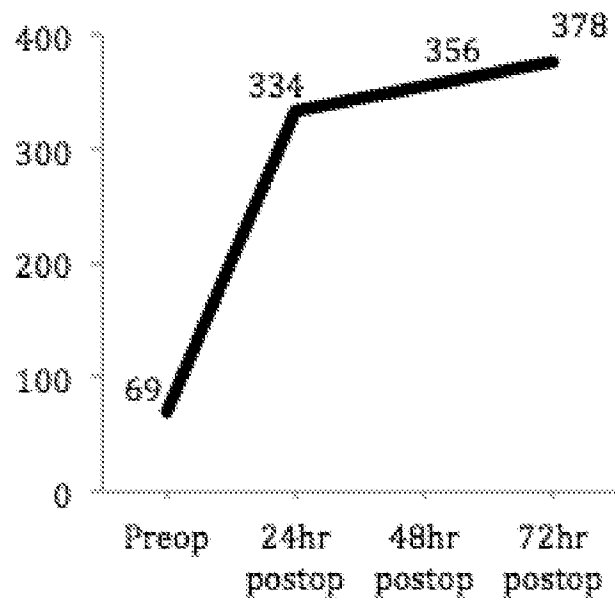


Figure 1B

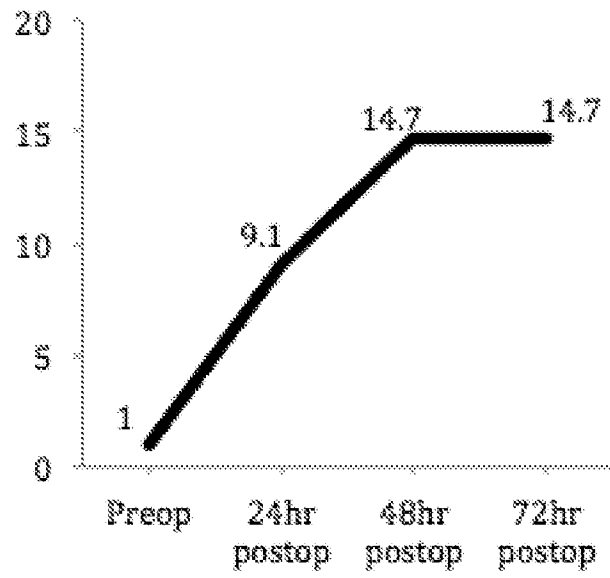


Figure 1C

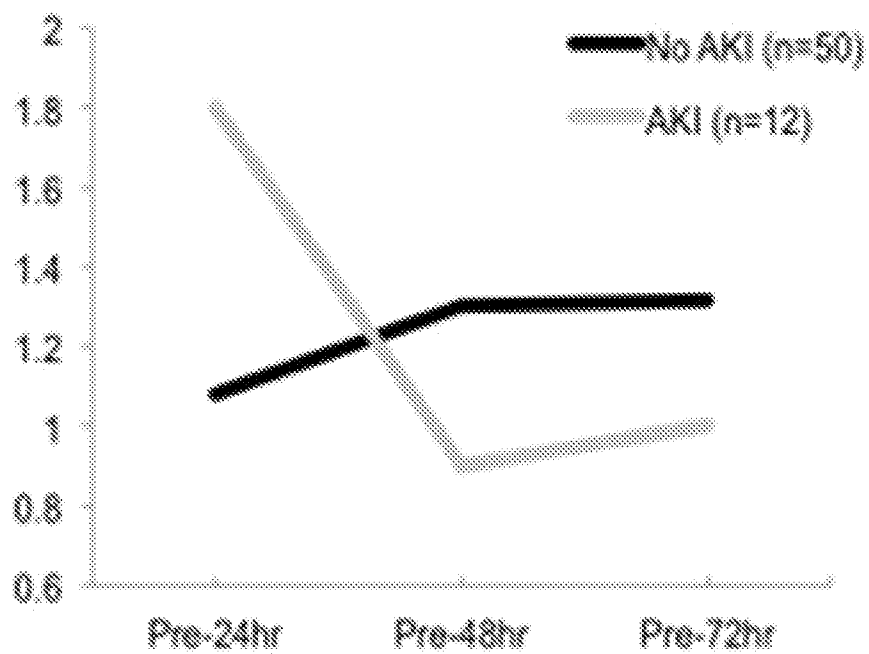


Figure 2A

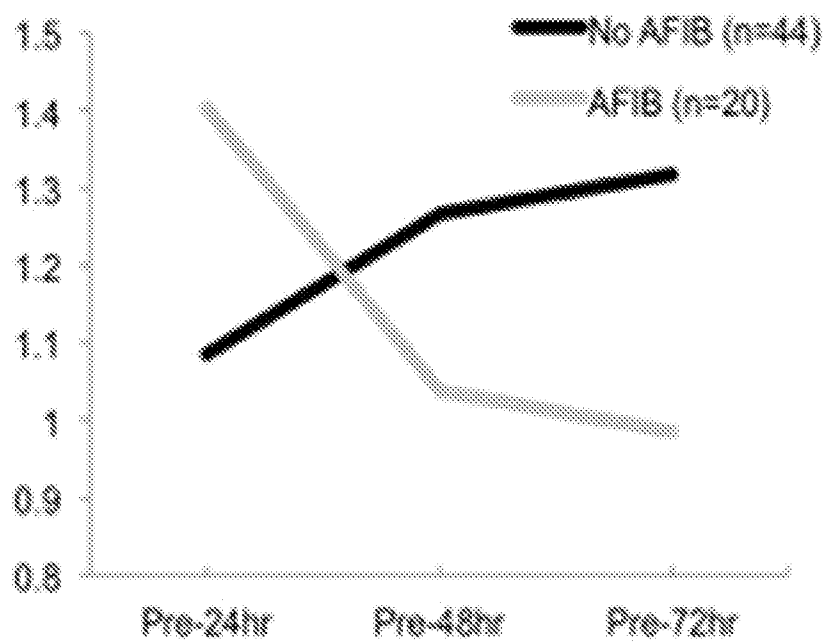


Figure 2B

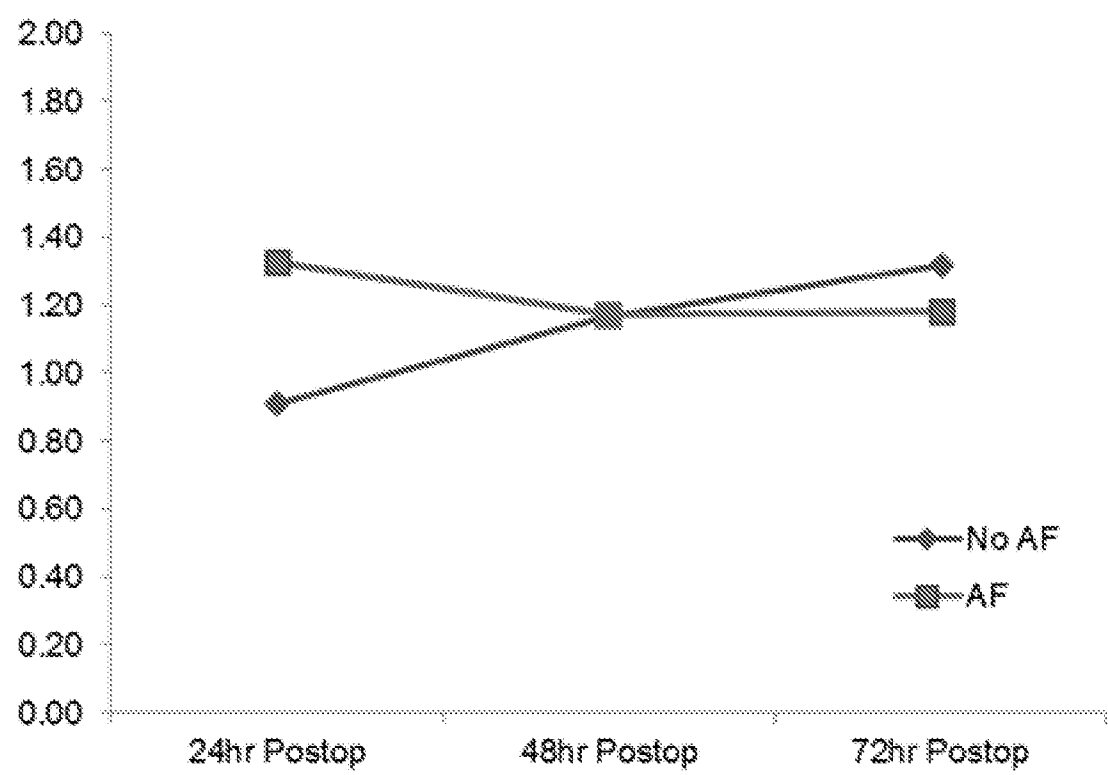


Figure 3

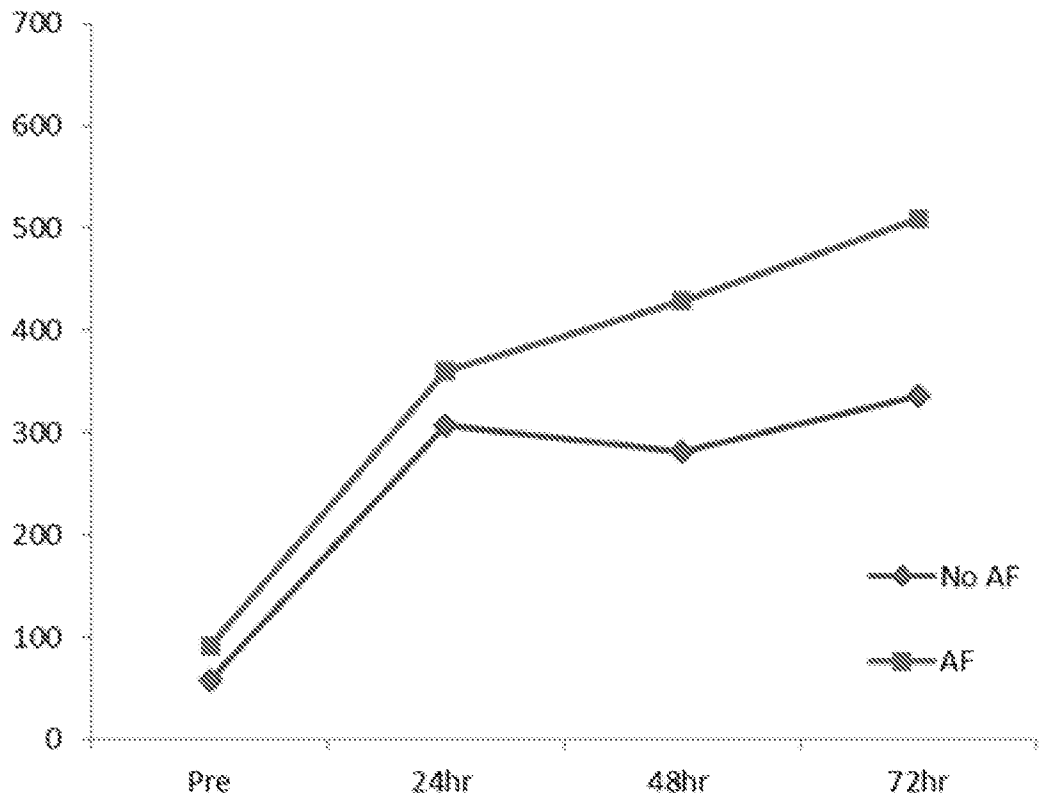


Figure 4

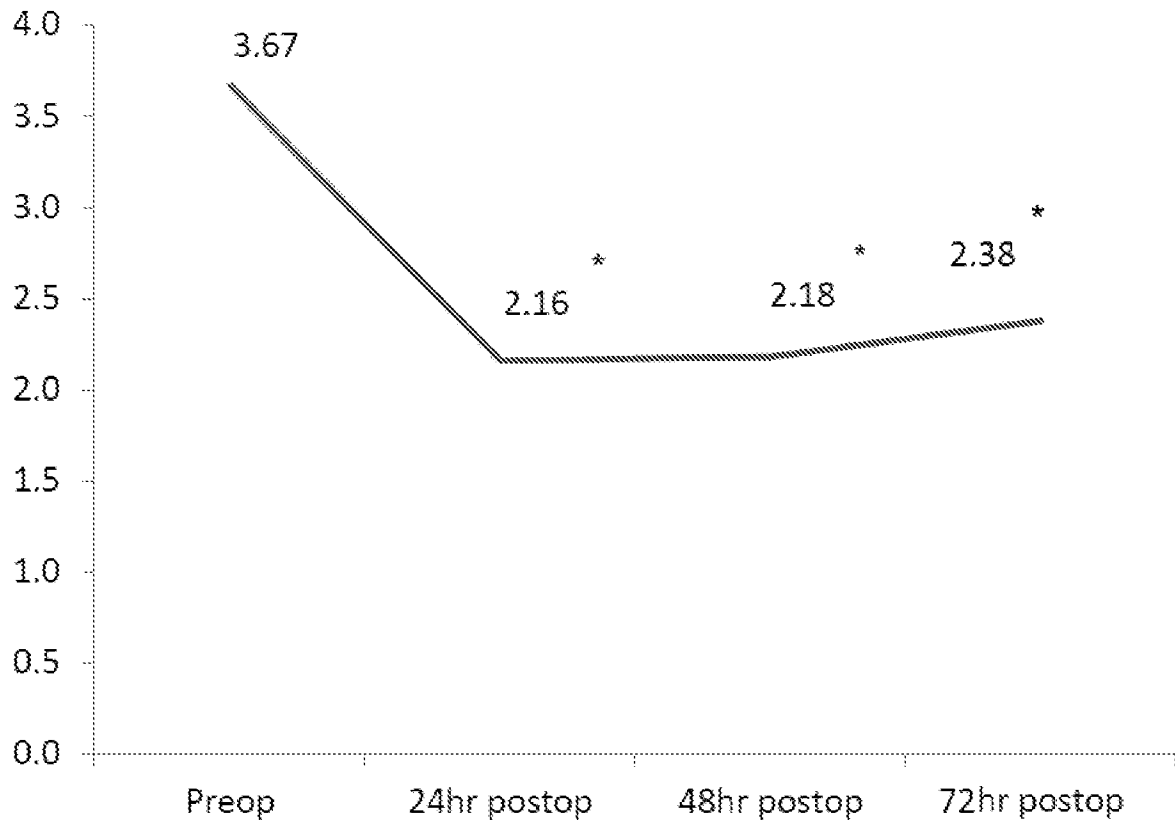


Figure 5A

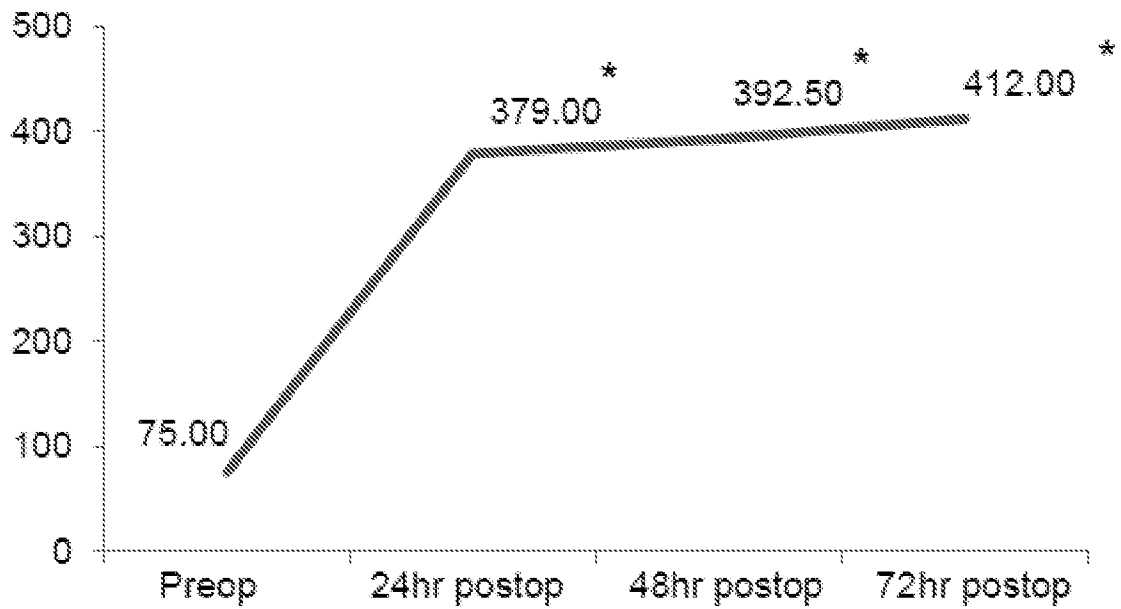


Figure 5B

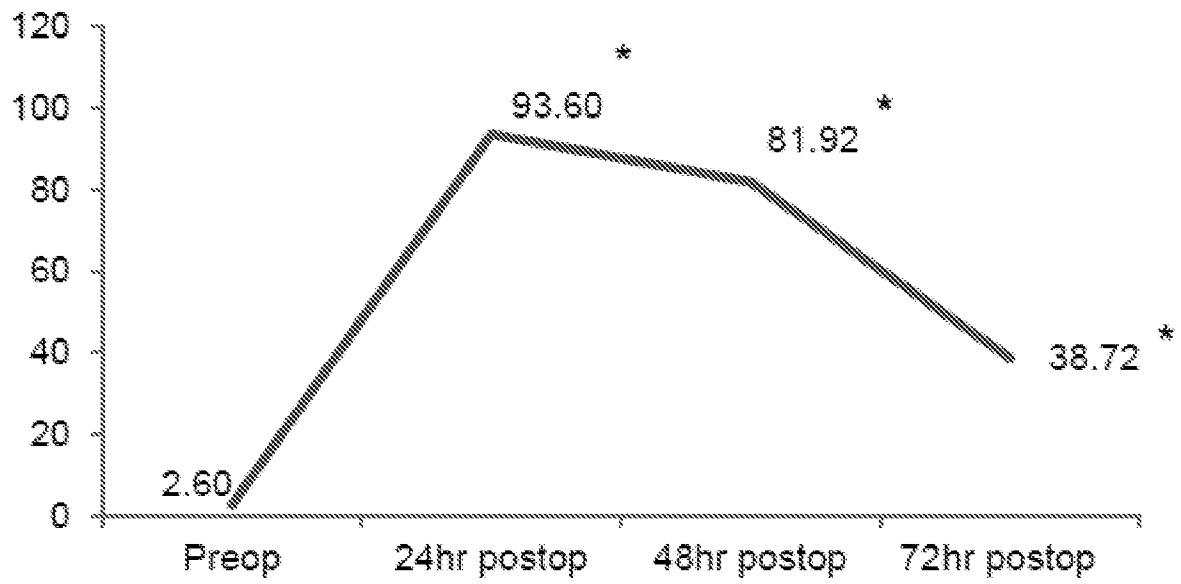
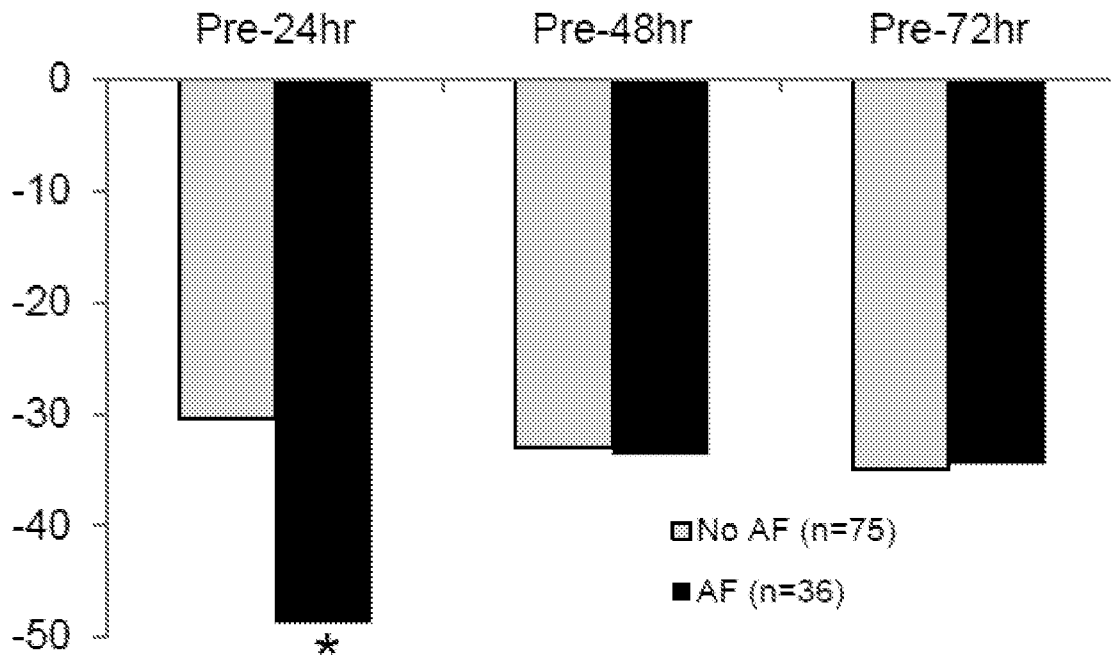


Figure 5C



NO % Change	Preop-24hr postop	Preop-48hr postop	Preop-72hr postop
No AF	-30.32	-32.98	-34.92
AF	-48.72	-33.65	-34.47
T-Test	p<0.01	p=0.7	p=0.5

Figure 6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US16/26590

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C01B 21/24; A61B 5/145; G01N 33/49 (2016.01)

CPC - C01B 21/24; A61B 5/145, 5/150755; G01N 33/0037, 33/4925

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) Classifications: C01B 21/24; A61B 5/145; G01N 33/49 (2016.01)

CPC Classifications: C01B 21/24; A61B 5/145, 5/150755; G01N 33/0037, 33/4925

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); Google Scholar; Google; EBSCO
diagnosis, nitric oxide, nitrite, nitrate, blood, serum, acute kidney injury

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	(UYSAL, A et al.) Perioperative high-dose amiodarone elevates nitric oxide levels in patients undergoing coronary artery bypass surgery. Biomedical Research. 2013. Vol. 24. No. 4. Pages 486-492; page 486, left column, 1st paragraph; page 487, right column, 4th paragraph; page 488, left column, 2nd paragraph; table 2	1-6, 7/1-3, 8/4-6, 9/1-3, 10/4-6
A	(CAI, H et al.) Downregulation of endocardial nitric oxide synthase expression and nitric oxide production in atrial fibrillation potential mechanisms for atrial thrombosis and stroke. Circulation. 2002. Vol. 106. Pages 2854-2858; page 2854, 'methods and results', left column, 1st paragraph; page 2855, left column, 2nd paragraph; page 2856, left column, 2nd paragraph; page 2857, right column, 3rd paragraph	1-6, 7/1-3, 8/4-6, 9/1-3, 10/4-6
A	(RATAJCZAK-WRONA, W et al.) Levels of biological markers of nitric oxide in serum of patients with squamous cell carcinoma of the oral cavity. International Journal of Oral Science. 2013. Vol. 5. Pages 141-145; page 142, right column, 1st-2nd paragraphs, left column, 5th paragraph	1-6, 7/1-3, 8/4-6, 9/1-3, 10/4-6
A	WO 2013/098294 A1 (L'UNIVERSITE CATHOLIQUE DE LOUVAIN) 4 July 2013; claim 1	1-6, 7/1-3, 8/4-6, 9/1-3, 10/4-6

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

7 June 2016 (07.06.2016)

Date of mailing of the international search report

12 JUL 2016

Name and mailing address of the ISA/

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Authorized officer

Shane Thomas

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PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US16/26590

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 11-16
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.