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A new method for selecting calf pericardium for use in cardiac bioprostheses on the basis of morphological and mechanical criteria

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The durability of existing calf pericardium bioprostheses is limited by phenomena such as mechanical stress and calcification, the factors most frequently implicated in valve failure. Varying the preferred direction of the collagen fibers influences the mechanical behavior of the pericardial membrane. Given this possible variation, a strict control of the selection of the biomaterial employed in the construction of valve leaflets is essential, but a reliable method of selection has yet to be established.

This study describes the development of a new system of *in vitro* selection involving a hydraulic simulator that reproduces the mechanical behavior of pericardial membranes subjected to the stress of continuous flow.

By combining morphological criteria such as thickness and homogeneity with those of mechanical behavior, and by selecting paired samples from different parts of the pericardium, we obtained excellent mathematical fits. Linear regression analysis provided the mode of predicting the tensile strength in a given sample when this value had been determined in its twin. The upper zones of calf pericardium, corresponding to either right or left ventricle but at a distance from ligamentous structures, showed the best mean results at rupture (60 MPa) and permitted the most reliable prediction. The expected stress for an elongation of 30% was 1.12 MPa, as was previously observed, with a 95% confidence interval of between 1.11 and 1.14 MPa.

These trials, together with the careful selection of the pairs, should help to establish definitive selection criteria.

Introduction

The durability of the existing bioprostheses is limited and unpredictable on an individual basis. Failures due to mechanical stress [1–7], calcification [8, 9] and even early tears in the valve leaflets in the absence of calcification, have been reported [10]. The mechanical stress to which the pericardial membrane used in the construction of the leaflets is subjected depends on physical parameters such as extensibility, elasticity and resistance to tears, and is related to the arrangement of its collagen fibers. Varying the preferred direction of the collagen fibers of the pericardial membrane changes its anisotropic mechanical behavior, there being a clear relationship between the latter and the collagen architecture [11]. Given this wide variation, a strict quality control of the material to be selected for the construction of the leaflets of the bioprosthesis is necessary since, to a large degree, the durability depends on said factor.

The variability among pericardial sacs from different animals, and even from one region of a given sac to another, is well known [11]. Among the variables involved are the thickness, the degree of orientation and the preferred direction of the collagen fibers, and they can not be controlled as they depend on factors such as animal age, body weight, nutrition and breeding. Thus, it is necessary to determine the morphological features of the different regions of the pericardial sac and their position with respect to the heart in order to establish reliable selection criteria [12–14].

At the present time, there is no definitive selection criteria. When Braile *et al*. [12] took into account mechanical features such as rupture tension, elongation and tensile strength in combination with the histopatho-

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logical features, they observed a better histological preservation of the elastic and collagen fibers of the pericardium corresponding to right ventricle, although the results of the statistical analysis show that bovine pericardium does not present sufficiently marked regional differences to identify a specific area for use in bioprosthesis construction. Using small-angle light scattering, a nondestructive optical technique to quantify the collagen fiber architecture of soft tissues, neither Sacks *et al*. [11] nor Hiester and Sacks [13–15] were able to establish conclusive selection criteria. A theoretically optimal region for selection should have a sufficiently large area of uniform thickness, negligible amounts of fat and a homogeneous preferred direction of the fibers. Our work was based on the hypothesis that in order to properly select bovine pericardium, it is necessary to combine anatomical or morphological parameters, such as membrane thickness and degree of homogeneity, with parameters of resistance or mechanical behavior.

Combining criteria of this type and analyzing a series of samples from a given zone within said region (corresponding to either right or left ventricle) enables us to characterize the mechanical behavior of the pericardium being assayed and, thus, predict that of its twin sample. For this study, we have employed a hydraulic model developed in our laboratory to reproduce the behavior of a pericardial membrane subjected to the stress of hydrostatic pressure. The final objective of this work is to develop a new *in vitro* system involving a hydraulic simulator to establish criteria for the selection of biomaterials that allow the prediction of their mechanical behaviors.

Material and methods

Calf pericardium obtained directly from the local abattoir was transported to the laboratory in cold isotonic saline (0.9% sodium chloride). Once the tissue was cleaned, each sac was mounted loosely on a 15 cm diameter ring, with the diaphragmatic attachment in the center and the sternopericardial ligaments on the circumference. Ten circular membranes measuring 2 cm in diameter were cut out of each sac, five from the pericardium enclosing right ventricle (region B) and five from that encasing left ventricle (region C), in such a that they formed five equivalent and symmetrical pairs according to the diagram in Fig. 1.

All the membranes were treated for 24 h with 0.625% glutaraldehyde prepared from a commercial solution of 25% glutaraldehyde (Merck) in 0.1 M sodium phosphate buffer (pH 7.4) at a ratio of 1/50 (w/v). The chemical treatment was applied to relaxed, tension-free tissue. The trial involved 5 series of 12 pairs from the aforementioned zones, for a total of 120 specimens.

The thickness of each membrane was determined by serial readings at 10 different points using a digital Mitutoyo micrometer (Elecount series E:A:33/8) with a precision at 20 °C of $\pm 3\,\mu$. Each membrane was subjected to increasing stress until rupture, which was accompanied by a loss of stress and confirmed macroscopically by the presence of a tear in the tissue.

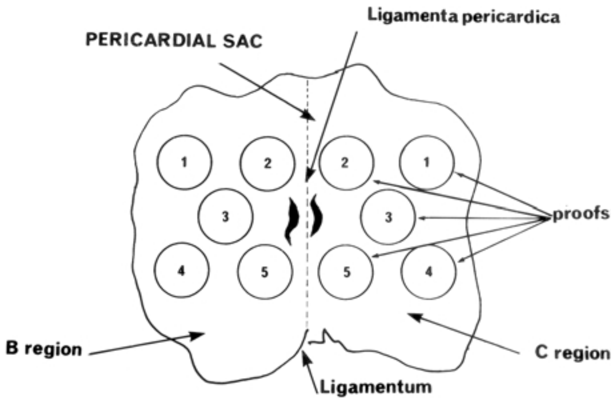

Figure 1 Illustration of the pericardial sac employed in the trial. Five symmetrical pairs of circular membranes are obtained for a total of ten specimens, one each from the following zones of the regions corresponding to right ventricle (B) and left ventricle (C): upper outer (zone 1), upper inner (zone 2), central (zone 3), lower outer (zone 4) and lower inner (zone 5).

Assay method

The assay was carried out on a hydraulic simulator capable of delivering increasing stresses to the pericardial membranes secured with pressure clips (Figs 2 and 3). The membranes were exposed to increasing hydrostatic pressure caused by the compression transmitted to saline solution by a piston. As the piston moved, the fluid deformed the membrane and a pressure gauge determined the pressure, ranging between 0 and 16 atmospheres. The simulator consisted basically of a unit for measuring pressure equipped with a servomotor to drive the pump propelling the piston. The entire system is illustrated in Fig. 4.

General description of the function of the system

A piston was activated by means of a digital monitor based on a high-speed processor that controlled the direct current electric servomotor. The piston compressed the fluid and the pericardial membrane resisted the pressure. The biomaterial was subjected to continuous, increasing pressure until rupture. The controlling computer indicated the angular velocity of the activating system, which was maintained throughout the trial. The data acquisition system evaluated the fluid pressure and the movement of the piston at all times. The numerical data corresponding

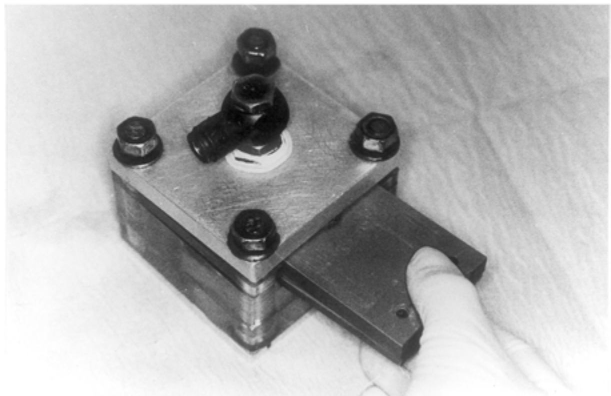

Figure 2 Clamping system to ensure the immobility of the samples during the trial.

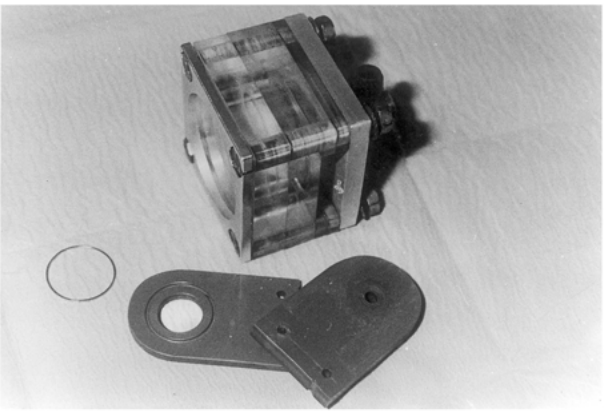

Figure 3 Introduction of the specimen into the clamp.

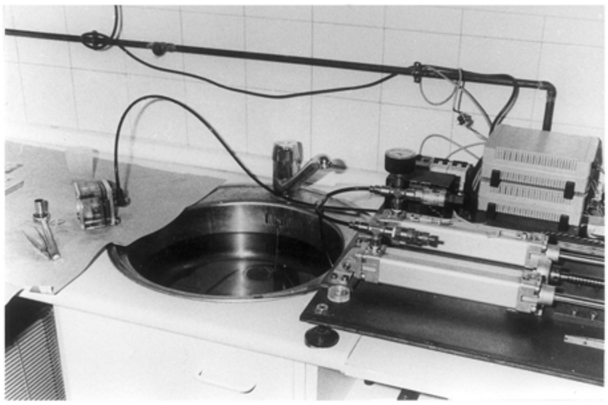

Figure 4 View of the equipment. Pressure pistons.

to these variables were transferred to a computer via a series interface, where they were stored for subsequent analysis.

Tensile strength

Once the pressure withstood by the pericardial membrane at each instant of the trial was known. Its tensile strength was calculated using a formula similar to the Laplace equation, described by Timoshenko [16] for a thin-walled membrane subjected to pressure: $Ts = pr/2e$, where p is the pressure in kg/cm^2, r the radius of the spherical membrane expressed in cm, e the thickness of the membrane in cm and Ts the tensile strength in kg/cm^2. To express this value in MPa, we divided the result by 10.19.

Elongation

The movement of the piston indicated the variation in the fluid volume at every moment and for each different pressure applied and, thus, the changes in membrane geometry up to the moment of rupture. At that point, the shape was that of a round bonnet the base of which was a known circle (the frame on which the membrane to be tested was mounted). By measuring the changes in length of the longest arc of the bonnet, it was possible to determine the percentage of elongation at each moment of the trial.

Statistical study and mathematical analysis

Comparison of means at rupture

The mean values at rupture in the different regions and zones of the membranes were compared taking into account all the zones, as well as paired zones.

Mathematical fit of the tensile strength/elongation ratio

The tensile strength (MPa)/elongation (per unit) ratio was studied using the least squares method. The best fit corresponded to a third-order polynomial, the shape of which is expressed as $y = b_1x + b_2x^2 + b_3x^3$, where y is the tensile strength in MPa and x is the per unit elongation of the membrane (the value of the constant b_0 was made to equal zero since due to biological considerations, the equation must pass through the origin. For these same considerations, the analysis was done for $x < 1$ (the behavior of the function when the membrane had surpassed its elastic limit, entering the realm of irreversible elongation, that is deformation, was not considered to be of interest).

Mean overall fit for the two regions (B and C) and the five zones (1, 2, 3, 4 and 5)

The tensile strength/elongation ratio was also studied, using the values obtained for each region and each zone within the regions.

TABLE I Mean values at breaking point (MPa)

Region (zones)	Number	Breaking point (MPa)	Standard deviation
B			
1	12	61.18*	12.65
2	12	29.13	10.00
3	12	28.77	5.72
4	12	39.99	9.96
5	12	48.43	13.72
C			
1	12	60.50	13.39
2	12	43.40	11.10
3	12	37.93	8.34
4	12	42.29	12.28
5	12	37.96	7.85

*Statistically significant ($p < 0.01$) with respect to the remaining values.

TABLE II Overall fit for zones 1, 2, 3, 4 and 5 of regions B and C

Region	b_1	b_2	b_3	R^2
B				
1	5.74	− 12.26	17.97	0.90
2	10.78	− 23.67	32.60	0.9.
3	9.30	− 15.53	25.24	0.92
4	6.57	− 10.88	19.54	0.93
5	7.47	− 14.10	22.22	0.96
C				
1	8.90	− 18.58	26.95	0.96
2	9.81	− 16.51	26.74	0.91
3	7.44	− 14.99	21.70	0.84
4	8.05	− 14.17	23.37	0.94
5	7.92	− 12.57	21.75	0.94

$y = b_1x + b_2x^2 + b_3x^3$, where y is expressed in MPa, x is the per unit elongation and b_1, b_2 and b_3 are the coefficients of the third degree parabola that fits the function (stress/elongation). R^2: determination coefficient.

TABLE III Selected mean overall fit for zones 1, 2, 3, 4 and 5 of regions B and C

Region	b_1	b_2	b_3	R^2
B				
1	5.48	− 10.97	17.17	0.97
2	10.58	− 23.06	32.79	0.98
3	7.24	− 12.81	21.39	0.94
4	6.34	− 9.03	18.42	0.94
5	7.41	− 14.30	22.23	0.96
C				
1	7.28	− 13.15	21.01	0.98
2	8.48	− 12.48	23.34	0.92
3	5.00	− 10.56	15.17	0.95
4	8.92	− 17.92	26.00	0.93
5	8.44	− 16.79	25.06	0.98

$y = b_1x + b_2x^2 + b_3x^3$, where y is expressed in MPa and x is the per unit deformation.

Selection criteria

Selection criteria were established to ensure greater homogeneity of the samples. The purpose of these statistical selection criteria was to determine the probability that each membrane tested actually belonged to the region or zone to which it was assigned in the initial selection. Thus, those membranes with a mean thickness that fell outside the 5th and 95th percentiles of the corresponding series were excluded, as were those membranes in which the difference between the mean and minimum thicknesses was greater than the mean value for this difference between the mean and minimum thickness was greater than the mean value for this difference determined for the corresponding series, plus one standard deviation. The pairs of membranes (one each from regions B and C) in which the stress (MPa) for $x = 1$ of region C was above or below the standard deviation of the mean for said value in region C were also excluded (it is assumed that the value for samples from B would be unknown and would be assigned to B in a future selection in which region C would be assayed and region B would be subject to selection).

Mean overall fit of the selected regions and zones

On the basis of the aforementioned criteria, the sample pairs selected from regions B and C were as follows: zone 1 (upper outer zone), pairs 2, 3, 4, 6, 7 and 11; zone 2 (upper inner zone), pairs 2, 4, 5, 7, 9 and 12); zone 3 (central zone), pairs 1, 3, 6, 9 and 10; zone 4 (lower outer zone), pairs 4, 5, 6, 7, 8 and 10; and zone 5 (lower inner zone), pairs 2, 4, 5, 7, 8, 11 and 12.

Predictive study (predicting the values for region B on the basis of those for region C)

A predictive study of the values for region B was performed after the selection process had been carried out and the values for the equivalent region C samples were known. This determination involved the mathematical calculation of the values for the selected pairs according to the aforementioned criteria, using linear regression analysis, where the values for region C were known independent variables and those of region B were predictive, dependent variables. The stress (MPa) in region B(y_b) was estimated on the basis of that of region C(y_c), and the 95% confidence intervals were calculated.

Results

Rupture

The mean values of the present series at rupture are shown in Table I. The upper outer zones (zone 1) of both regions B and C presented the best results (61.18 MPa and 60.50 MPa, respectively), showing no statistically significant differences between them. The differences between the values for this zone and those of all the

TABLE IV Coefficients (b_0 and b_1) for the predictive study using linear regression analysis to determine the behavior of samples from zones of region B on the basis of region C specimens

Zone	b_0 95% CI	b_1 95% CI	R^2
1	0.01 (− 0.01, 0.21)	0.71 (0.70, 0.72)	1.000
2	0.11 (− 0.01, 0.24)	0.93 (0.84, 1.01)	0.992
3	− 0.05 (− 0.12, 0.01)	1.70 (1.61, 1.79)	0.997
4	− 0.08 (− 0.18, 0.02)	0.93 (0.85, 1.01)	0.992
5	− 0.01 (− 0.03, 0.003)	0.91 (0.89, 0.92)	1.000

$y = b_0 + b_1 x$
y = MPA; x = per unit elongation

TABLE V Predictive study (B f(C)) zone 1: estimation of the values for stress (y_o)

Elongation	Stress (MPa) y_b (real)	Stress (MPa) y_c (real)	Stress (MPa) y_b (predicted)	95% CI
0.05	0.25	0.33	0.25	0.22, 0.26
0.10	0.46	0.62	0.45	0.43, 0.47
0.15	0.63	0.87	0.63	0.61, 0.64
0.20	0.79	1.10	0.79	0.77, 0.81
0.25	0.95	1.33	0.95	0.93, 0.97
0.30	1.12	1.57	1.12	1.11, 1.14

Per unit elongation. 95% CI: 95% confidence interval.

remaining zones were statistically significant ($p < 0.01$). Samples from the other zones reached breaking point at mean values ranging between 28.77 MPa and 48.43 MPa; there were no statistically significant differences among them.

Mathematical fit of the tensile strength/elongation ratio

The individual equations for each region and zone showed an excellent fit to cubic parabolas ($R^2 > 0.95$). Table II shows the results of the mean overall fit for zones 1, 2, 3, 4 and 5 of regions B and C. After applying the selection criteria described in the Material and methods section, the mean overall fit appears in Table III, which shows a clear improvement in the correlation coefficients. Fig. 5 describes the pressure/volume relationship, the fluid is desplaced by pericardium deformation.

Predictive study

This study was designed to predict the behavior, in terms of the values for stress (MPa), of samples from region B(y_0) on the basis of that of samples from region C(y_c). The 95% confidence intervals were also calculated. Linear regression analysis of the behavior of the tissue from each of the five zones of each region showed an excellent fit, with correlation coefficients > 0.99 in every case. These results are displayed in Table IV.

The results of the prediction in one zone, for a per unit elongation of between 0.05 and 0.30, are shown in Table V.

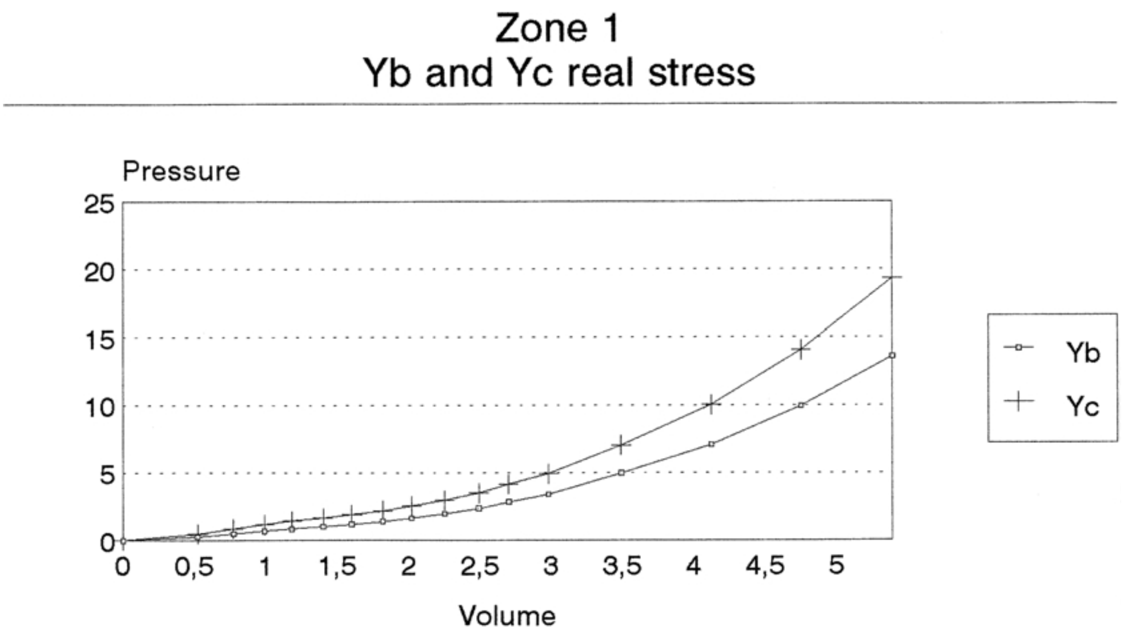

Figure 5 Pressure/volume relationship, pressure in MPa and volume in cm^3.

Discussion

The variation in the distribution of elastic and collagen fibers of the pericardial tissue is related to the consistency of the material, producing areas of greater or lesser resistance (12). This anatomic variability in terms of the preferred direction of the collagen fibers makes it difficult to determine sites for tissue selection and may have a negative influence on the durability of the cardiac prostheses constructed with biological materials. Numerous studies have focused on the attempt to find areas of the pericardial sac of homogeneous structure and large enough for use in the construction of cardiac valve leaflets, but conclusive selection criteria have yet to be defined [11–15, 17]. In 1994, Sacks *et al*. reported that the region of pericardium protecting left ventricle was that most homogeneous and suitable for selection [11]. More recently, in 1998, Hiester *et al*. [13, 14] recognized that the selection of the tissue on the basis of anatomy alone did not guarantee its suitability and that the measurement of fibers for the classification and selection of the pericardium was a questionable approach. One thing that has been demonstrated is the quantitative relationship between the collagen fiber architecture and the anisotropic mechanical behavior. The variability of this behavior is caused mainly by changes in local fiber preferred directions [11]. For a careful preselection of the material to be employed in the construction of cardiac valve leaflets, it must be possible to make a reliable prediction of its mechanical behavior, including its anisotropy. Truly engineered designs will be necessary to solve the problems of bioprosthesis durability [14]. The aim of our study is to contribute to this effort.

The use of paired, theoretically twin, samples fulfilling both morphological criteria (thickness, homogeneity) and mechanical criteria (absorbed stress within the elastic limit) responds to the challenge of designing a bioprosthesis based on engineering concepts [18]. The good behavior of the bioprostheses used to replace tricuspid valves, which perform a less mechanical activity [19], encourages the search for more resistant and uniform areas in the biomaterials employed.

The analysis of our results at breaking point show that the mean values of the upper outer zone (zone 1) of both regions B (corresponding to right ventricle) and C (corresponding to left ventricle) were very similar (61.18 and 60.50 MPa, respectively) and far superior ($p < 0.01$) to those recorded for the rest of the zones (Table I). Resistance to rupture is not the best parameter for the evaluation of biomaterials, a finding that was reported by Von Fraunhofer in 1988 [20]. Our trials show that there are zones with mean breaking stresses one third lower than those of other zones, a fact that invites reflection on the following assumption [10]: that perhaps the only portions of the membrane that should be utilized are those offering greater resistance and greater uniformity [14].

The analysis of the stress/elongation ratio (Tables II and III) demonstrates the better overall fit of each series of trials according to zone when the selection is based on criteria with excellent correlation coefficients. The application of linear regression analysis to these results (Table IV) enables the prediction of the tensile strength in region B, once that of its paired sample has been determined, and its comparison with the real value obtained. Table V shows the predicted values in one zone for an elongation of up to 30%. Obviously, a better prediction and a narrower confidence interval are obtained with better fitted linear regression curves (zones 1 and 5) although, of the two, we would choose zone 1 (Fig. 1), with its marked resistance to rupture and its distance from ligamentous structures (the pericardial ligament near zone 2 and the ligamentum near zone 5). Ligaments can distort the collagen architecture, changing the preferred direction of its fibers [13]. Should zone 1 be selected, the predicted stress for an elongation of 30% is 1.12 MPa, with a 95% confidence interval of 1.11 to 1.14 MPa, while the real stress for the same 30% elongation was 1.12 MPa, a prediction difficult to surpass in precision.

As the pericardium is a sac (Fig. 1 illustrates the open sac), the use of paired, nearly contiguous samples offers the advantage that one specimen can predict the behavior of its pair with a tiny margin of error. The repetition of these trials, together with the careful selection of the pairs, should help to establish definitive selection criteria. Having suggested a reliable approach to tissue selection, our next objective is to validate these criteria by means of dynamic fatigue testing (19), in the attempt to definitively confirm our hypothesis.

Appendix I

Technical features of the system

The most relevant technical specifications are as follows:

Amplifier: D-MOS technology; H-bridge configuration; maximum working voltage; 53 V; maximum intensity in steady state: 3 A.

Motor: Rated voltage: 24 DCV; rated output, 15 watts; starting torque, 120 mNm; current in a vacuum, 21.7 mA; starting current, 3040 mA; maximum permanent torque, 30.46 mNm.

Incremental position sensor: optical; two quadrature outputs and index impulse.

Quadrature processor: programmable logic technology; two quadrature inputs; incremental/decremental monopolar impulses output; maximum working frequency of 4 MHz.

Digital compensator: processor, RISC microcontroller, 24 MHz, 8 bits, 200 ns/instruction; maximum sampling frequency, 1 KHz; velocity range from 00 to 8 388 607 counts/sampling period $\times$ 256; proportional action coefficient (KP) of $-32\,768$ to 32 767; differential action coefficient (KD) of $-32\,768$ to 32 767.

Piston: 160 mm stroke; 32 mm in diameter; maximum pressure 16 atm.

Pressure sensor: maximum pressure 16 atm; output signal: 4–20 mA.

Computer: standard Pentium-75 configuration.

Appendix II

Calculation of the radius (R) of the curvature of a membrane subjected to stress. The following expressions were used:

$$V = \pi\left(Rh^2 - \frac{h^3}{3}\right) \quad (1)$$

$$V = \frac{\pi h r^2}{2} + \frac{\pi h^3}{6} \quad (2)$$

$$R = \frac{r^2 + h^2}{2h} \quad (3)$$

where V is the volume measured by the machine as a function of the distance the piston moves, r is the radius of the orifice over which the membrane is mounted and h is the height of the round bonnet formed as the membrane stretches.

Equation (2) provides h, resolving the third-order equation. R is obtained using Equation (3) (radius of the sphere containing the deformation of the membrane or leaflet). Knowing R and the pressure, it is possible to determine the stress exerted on the membrane (see Material and Methods).

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