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Rheological Behavior of Different Calf Sera before, during and after Biomechanical Testing

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Abstract: Due to different rheological behavior of human synovial fluid and the test mediums for in vitro examinations, wear tests cannot replicate the in vivo situation completely. The standards for wear testing indicate calf serum as in vitro test medium. However, these standards do not contain precise information on the main constituent components and the rheological properties. In this study, bovine calf serum and newborn calf serum with a protein concentration of 20 g/L, both approved for wear testing defined by the International Organization for Standardization (ISO), were characterized according to their rheological properties to detect differences before and during tribological simulation. The rheological behavior was determined at five defined intervals of a tribological test. The two test fluids differ in their rheological properties before and during the test and can therefore lead to deviating results in tribological testing. Furthermore, both test media changes considerably over test duration. At a test duration of 0.5 Mio cycles, both fluids have changed so that there is no longer any difference between them in terms of rheological properties. These changes could be attributed to denaturation and degradation of proteins. Thus, the choice of medium impacts tribological test results which should be considered for the interpretation of these studies.

Keywords: rheology; viscosity; lubricant degradation; calf-serum; wear

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1. Introduction

In 2020, more than 120,000 endoprosthetic joint replacement procedures were reported by the Australian Orthopaedic Association National Joint Replacement Registry [1]. The aim of endoprosthetic replacement is to restore the functionality of the joints and to ensure painless mobility for as long as possible [2]. However, in joint replacement, implant failure and revision also occur due to aseptic and septic loosening [3]. The types of failure are usually multifactorial, but the main reasons may be mechanical or biological nature [4–6]. The release of wear particles from the implant material, due to articulation of the artificial implant components, plays an important role [7]. Depending on the quantity, size, shape, and chemical composition of the wear particles, wear related revisions can occur [8–10].

In order to assess possible failure causes prior to implantation, tribological examination of implants used in total joint replacements is essential. Wear testing of joint replacements is well established and normatively defined for knee (ISO 14243) and hip arthroplasty (ISO 14242) [11–13]. It offers the possibility to predict long-term clinical performance by studying friction and wear behavior under in vitro conditions [14,15]. Nevertheless, the in vitro wear tests cannot completely represent the in vivo conditions. Some investigations have already shown that results from in vitro studies could not correspond to the in vivo situation with respect to particle characteristics [16,17] and wear behavior [18,19]. One

reason for these discrepancies could be the difference in tribological properties between in vivo and in vitro conditions due to another rheological behavior of human synovial fluid and the test medium for in vitro examinations [20,21]. The synovial fluid (SF) in the natural healthy human joint contributes to joint lubrication and supports bearing functions by reducing friction during movement and absorbing peak loads [22]. It is already known that an altered concentration of the compounds as well as the different rheological properties of the degenerated synovial fluid play a decisive role in wear behavior of the human joint [23,24]. In this regard, the reduced viscosity compared to healthy SF is often in the focus of attention [25]. However, it is not possible to use SF in standardized in vitro tests. This is not ethically justifiable and may be unsuitable in terms of comparability due to the small amount of SF that can be removed and the high variation in rheological properties between different patients. For these reasons, it is necessary to use a replacement fluid that has similar properties to imitate human SF. A major challenge in defining a suitable fluid is that the human SF regenerates permanently and therefore achieves approximately constant properties over time after endoprosthetic treatment. Particularly as synthetic fluids cannot regenerate themselves, they should nevertheless exhibit consistent properties over the duration of tribological investigations.

Any change in the fluid properties, which causes different lubrication conditions and wear behavior in tribological testing, can lead to an inadequate reflection of the in vivo situation [16,18]. For this reason, the properties of test fluids were specified in standards for wear simulators [11,12]. The standards indicate calf serum as fluid test medium because the wear mechanisms and the debris morphology are all comparable to clinical performance. The protein concentration and other chemical additives are defined to emulate human synovial fluid as closely as possible, to prevent bacterial growth and to minimize calcium phosphate films on the implant surface. For standardized wear tests of knee endoprostheses a protein concentration of 20 g/L [12] and for hip endoprostheses 30 g/L is used [11]. However, these standards do not contain precise information on the main constituent components, such as the concentrations for albumin, γ-globulin, phospholipids, or hyaluronic acid. The desired rheological properties are also not further defined. A few studies have therefore examined the rheological properties of bovine serum and possible factors influencing them [26,27]. However, these studies only deal with a specific medium, the bovine calf serum (BCS). Due to the already mentioned lack of definition of the used serum, other fluids, such as newborn calf serum (NBCS), are available and approved for biomechanical testing according to the existing standards. When preparing the fluid to a protein concentration of 20 g/L, other ingredients can vary between NBCS and BCS. Whether this difference influences the rheological properties and the wear behavior remains elusive.

In addition to the protein concentration of the fluid, a complete replacement of the test fluid at least every 0.5 million cycles is specified in the standards. However, it has already been proven that shortening this replacement interval leads to an increase in wear [28]. This can probably be explained by the denaturation of the contained proteins during the test run. While continuous regeneration of proteins occurs in vivo, this is not possible in vitro. Furthermore, it was claimed that the denatured proteins could increase the adhesive wear response [29]. Visual changes in the fluids can also be reported from previous tests. If the test fluids are compared optically before and after the test over 0.5 million cycles, a clear turbidity of the fluid after the test run can be seen [30]. In addition, precipitations can be observed on the sliding surfaces, and if the fluids are left to stand for a longer period after the test, a viscous phase also settles. Nevertheless, it has not yet been considered if denaturation of proteins also influences the rheological behavior of the test fluids.

The aim of this study is to characterize the rheological properties of newborn calf serum and to compare them directly with the already investigated bovine calf serum regarding its suitability in biomechanical testing. In addition, it will be investigated whether the rheological properties of the test fluids change over the duration of test intervals due to the denaturation of the proteins. These aims lead to the following two main questions:

1. Do NBCS and BCS show different rheological properties?
2. Do the rheological properties of the two fluids change after different testing intervals?

2. Materials and Methods

2.1. Test Fluid

The first test fluid is the bovine calf serum (BCS), which has already been investigated rheologically [26,27]. The BCS of the company Biochrom GmbH (Berlin, Germany) has a protein content of 72.3 g/L in the raw serum. As a comparison medium the newborn calf serum (NBCS) from PAN-Biotech GmbH (Aidenbach, Germany) with a protein content of 78.9 g/L in the raw serum was examined. The exact composition of the two sera is compared in Table 1.

Table 1. Composition of bovine calf serum (BCS) and newborn calf serum (NBCS) as specified by the supplier.

	Unit	BCS (Raw)	NBCS (Raw)
Parameter/Biochemical Assay			
Cholesterol	[mg/100 mL]	138.1	167.5
Triglycerides	[mg/100 mL]	8.9	14.6
Glucose	[mg/100 mL]	110.1	81.3
Total protein	[mg/mL]	72.12	78.95
Capillary Electrophoresis			
Albumine absolute	[mg/mL]	32.90	36.63
α-Globuline absolute	[mg/mL]	15.80	17.68
β-Globuline absolute	[mg/mL]	10.20	8.13
γ-Globuline absolute	[mg/mL]	13.20	16.50
Albumin/Globulin-quotient	n.a.	0.84	0.86
Other			
pH value	n.a.	7.81	8.03
Osmolality	[mOsm/kg]	300	299
Hemoglobin	[mg/100 mL]	14.7	22.7
Endotoxin	[EU/mL]	2.6	9.256

n.a.: not available.

To achieve properties corresponding to the joints, the test fluids were first diluted by adding deionized water. For direct comparison of the two test fluids a protein content of 20 ± 1 g/L for knee joint replacements [12] were set. Sodium azide (NaN_3) (1.85 g/L) and 5.85 g/L ethylenediaminetetraacetate (EDTA) were added as anti-microbiological additives to both test fluids to prevent bacterial growth and to minimize calcium phosphate films on the implant surface [11,12]. After preparation, all samples were pre-cooled to 4 °C and were deep frozen afterwards at −20 °C for two to four months. Before starting the test, the respective serum was slowly thawed to 8 °C and stored at room temperature.

2.2. Test Setup

The biomechanical test is carried out using a servo-hydraulic knee joint simulator KS2-6-1000 (AMTI, Watertown, MA, USA) with four active degrees of freedom and a programmable virtual soft tissue model (Figure 1). The simulation parameters were implemented according to ISO 14243-1:2009 [12]. The test parameters are described in detail in previous studies [7,31].

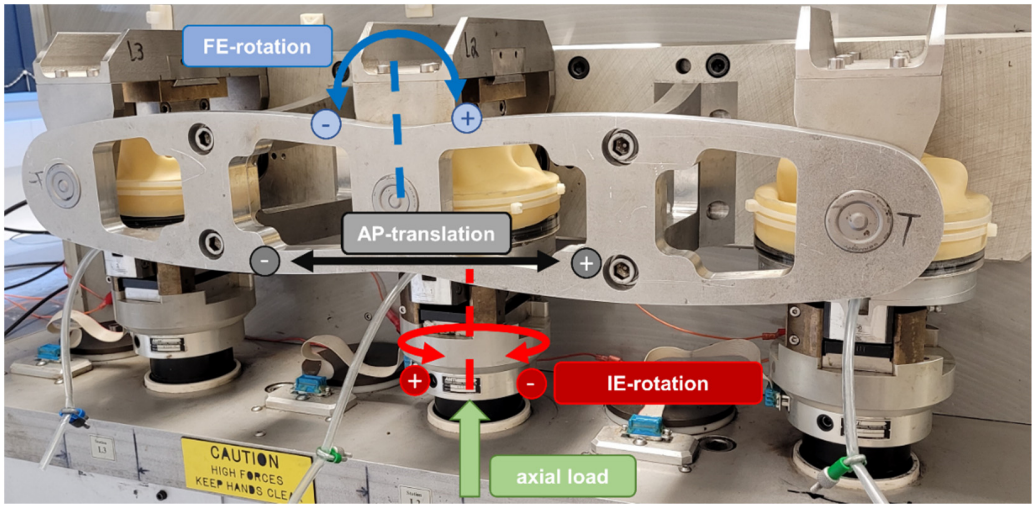


Figure 1. Test setup with the axis of rotation and translation of knee movement; FE = flexion/extension, AP = anterior/posterior, IE = internal/external.

Four Attune implant systems (DePuy Orthopaedics, Inc., Warsaw, IN, USA) were used as test components, each consisting of a cruciate retaining femoral and a fixed bearing titanium tibial tray in size 5. A crosslinked polyethylene insert size 5 blended with antioxidants and a thickness of 5 mm was selected. The inserts were pre-soaked. The biomechanical test was performed in closed chambers with 250 mL test fluid at a constant temperature of 37 ± 2 °C in each chamber. The frequency of the simulation for 1 cycle was 1 ± 0.1 Hz.

For both test fluids, knee simulations of increasing durations were performed. Thus, the tested serum was replaced after the intervals 1000, 10,000, 0.1 × 10^6, 0.5 × 10^6, 1.0 × 10^6 cycles. The collected test fluid was directly frozen.

2.3. Density Measurements

Density measurements were performed using a DMA 4500 density meter (Anton Paar GmbH, Graz, Austria). According to the technical specifications, this measuring system provides an accuracy of 0.00005 g/cm^3 and a digital resolution of 0.00001 g/cm^3. Density measurements were carried out for both test fluids before biomechanical testing (0 cycles).

2.4. Rheologic Behavior

The rheologic behavior of the two test fluids was determined using a Physica MCR 702 rheometer (Anton Paar GmbH, Graz, Austria). The rheological investigations were carried out with a double gap measuring system at a temperature of 37 ± 0.04 °C. The dynamic viscosity was measured as a function of the shear rate in a range from 10 s^{-1} to 1000s^{-1}. Here, the increase occurs in 15 logarithmic steps, starting with low shear rates. The exact specifications of the rheometer and the preparations before the start of the rheological examination can be found in the previously published preliminary study [26].

Measurements with repetition of n_{NBCS} = 10 using fresh samples each were performed for NBCS with protein concentration of 20 g/L to demonstrate comparability to BCS. In addition, BCS and NBCS were examined at different cycle numbers. Here, each setting point was measured three times using fresh samples. After all measurements were completed, two samples of NBCS after a test interval of 1.0 × 10^6 cycles were placed in a temperature-controlled measurement room (21 °C) for 24 h to provoke a segmentation of the severely degraded and denatured proteins. This produces 3 optically distinguishable phases, which were removed and rheologically analyzed. Each fluid was measured three times using fresh samples. Additionally, to ensure that the tests performed are comparable to the literature, the measurements were verified through preliminary tests with deionized water at 30 °C

(n_{water} = 3). At this temperature, the viscosity of deionized water ($\eta_{water,30°C} = 0.8$ mPa·s) is in the range of observed viscosities of the test fluids at higher shear rates.

Furthermore, the Reynolds number was calculated for all measurements to ensure laminar flow for the entire shear rate range. In the annular gap of a cylinder measuring system, the Reynolds number was generally calculated as

$$Re = \frac{v_m \cdot L \cdot \rho}{\eta} \quad (1)$$

with ρ being the density of the fluid, η the measured dynamic viscosity, L the annular gap and v_m the velocity referring to the gap center. The annular gap can be determined from the difference between the outer and inner radius of the test setup.

$$L = R_e - R_i \quad (2)$$

The velocity, which is referred to the center of the gap, was determined as follows

$$v_m = \omega \cdot \frac{R_e + R_i}{2} \quad (3)$$

with ω being the angular velocity [32].

2.5. Protein Measurements

To ensure comparable protein concentrations of the test fluids, samples of the test fluids before biomechanical testing (0 cycles) were analyzed using a modified Biuret reaction method with the Dimension® EXL™ 200 (Siemens Healthace GmbH, Erlangen, Germany), in the in-house laboratory. In addition, the three evoked distinguishable phases of NBCS after a test interval of 1.0×10^6 cycles were re-examined for protein concentration using the same method. Here, for each evaluation, three analyses were performed with 2 mL of each sample.

2.6. Statistical Analysis

A descriptive analysis containing arithmetic mean, standard deviation and 95% confidence interval is given for all results. Shapiro–Wilk test was used to confirm the normal distribution of the data. Afterwards, a repeated measures Analysis of Variance (ANOVA) of independent variables was applied. To compare the two different test fluids before testing and at the different cycle numbers of each serum, an ANOVA with a Bonferroni correction was used. The level for statistical significance was set at $p < 0.05$. The software SPSS Statistics (Version 25, IMB Inc., Armonk, NY, USA) was used for statistical analysis.

3. Results

3.1. Optical Observation

Figure 2 shows the optical progression of the two fluids BCS (Figure 2a) and NBCS (Figure 2b) over all six test intervals. The turbidity of the liquids was visually inspected to identify optical trends. Before the fluid is tested in the test setup, both, BCS and NBCS, are almost identically transparent. As the test duration increases, there is a steady increase in the turbidity of the medium. Compared to BCS, turbidity seems to occur earlier with NBCS. At interval 1000 cycles, BCS is still transparent, whereas NBCS is already becoming turbid when inspected visually. Nevertheless, even before biomechanical testing, there is a slight difference between the two sera. The state of turbidity seems to be irreversible and did not change without further mechanical stress.

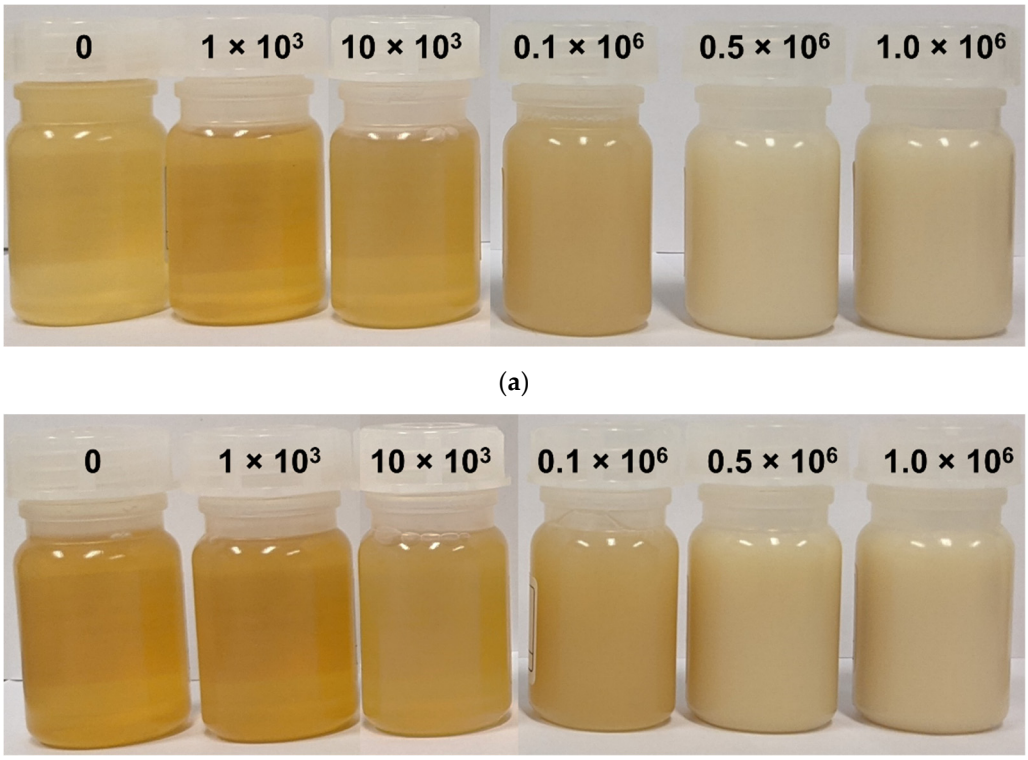


Figure 2. Optical progression of the fluids over test intervals: (**a**) bovine calf serum; (**b**) newborn calf serum.

3.2. Density Measurement

In Table 2, the results of the density measurements for two test fluids before tested in the test setup (0 cycles) are given. The densities of the two test sera differ significantly from each other ($p < 0.001$), with NBCS having a lower density than BCS. Nevertheless, the difference between the two measured densities is relatively small.

Table 2. Density of bovine calf serum and newborn calf serum at 37 °C—0 cycles.

Test Serum	Absolute Density [g/cm^3]
BCS	1.00441 ± 0.00001
NBCS	1.00420 ± 0.00001

3.3. Rheologic Behavior

In Figure 3, the results of the dynamic viscosity measurements before testing and after an interval of 0.5×10^6 cycles are given as boxplots. On the left side, the results for BCS and on the right side the results for NBCS are shown. Before biomechanical testing (0 cycles), both fluids clearly show a shear-thinning behavior merging into nearly Newtonian behavior at high shear rates ($\gamma \geq 100\ s^{-1}$). A higher deviation of the measured values can be seen for low shear rates. For the typical test interval of wear simulation studies (0.5×10^6 cycles) a nearly Newtonian behavior can be observed over the entire shear rate range. Furthermore, the standard deviation is lower over the complete measurement range. Above a shear rate of $\gamma \geq 371\ s^{-1}$, a tendency towards shear-thickening behavior can be observed at the interval of 0.5×10^6 cycles. However, for small shear rates, a slightly shear-thinning behavior still can be seen.

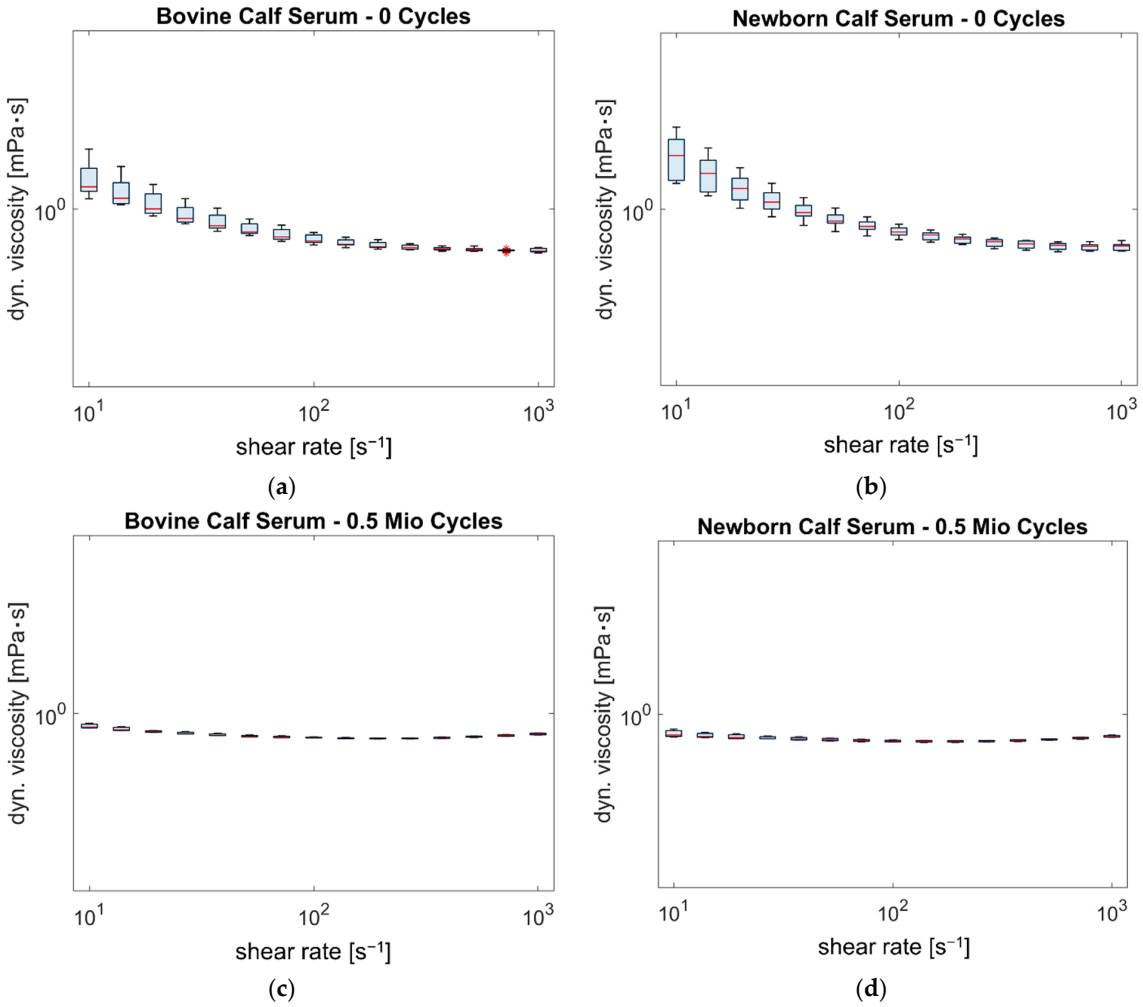


Figure 3. Dynamic viscosity over shear rate. (**a**) bovine calf serum—0 cycles; (**b**) newborn calf serum—0 cycles; (**c**) bovine calf serum- 0.5×10^6 cycles; (**d**) newborn calf serum—0.5×10^6 cycles. * = outliners.

Table 3 summarizes the mean values and 95% confidence intervals of the two measured test fluids at all test intervals at selected shear rates, on which the statistical evaluation is based.

The measurements of the two test sera before the test (0 cycles) and after a test interval of 0.5×10^6 cycles were approximately normally distributed, as assessed by the Shapiro–Wilk test ($p > 0.05$). A repeated measures ANOVA with a Greenhouse–Geisser correction showed that there is a statistically significant difference between the different testing intervals for all test sera and water (0 cycles: $F(1.063, 21.252) = 60.634$, $p < 0.001$); 0.5×10^6 cycles: $F(1.304, 7.824) = 90.481$, $p < 0.001$). Bonferroni post hoc analysis revealed no statistically significant difference in the comparison of BCS and NBCS before the test ($p = 0.241$) and after a testing interval of 0.5×10^6 cycles ($p = 0.178$).

Figure 4 shows the comparison of the mean dynamic viscosity curves depending on the shear rate at different testing intervals for BCS (Figure 4a) and for NBCS (Figure 4b). Looking at BCS, a clear increase in dynamic viscosity can initially be identified for shear rates $\gamma \geq 51.3\ s^{-1}$ after 1000 cycles. In the interval range from 1000 to 0.1×10^6 cycles, a tendency of decreasing viscosity with increasing testing cycles can be seen. At testing intervals 0.5 and 1.0×10^6 cycles, this tendency is inverted, whereby an increasing viscosity with increasing cycles is detected. For shear rates $\gamma \geq 51.3\ s^{-1}$, the viscosity decreases with decreasing cycles up to a test duration of 0.1×10^6 cycles. Considering the test intervals of 0.5 and 1.0×10^6 cycles, the same tendency as for higher shear rates can be observed. For NBCS, the same tendency can be obtained for shear rates $\gamma \leq 51.3\ s^{-1}$ over all intervals. At

shear rates $\gamma \geq 51.3\ s^{-1}$, no clear trend in dynamic viscosity depending on the cycles can be determined in the interval range from 0 to 0.1×10^6 cycles. Only for the test cycles of 0.5 and 1.0×10^6 cycles, a clear tendency of increasing viscosity with increasing cycles can be observed again.

Table 3. Overview of dynamic viscosity at specific shear rates represented by mean value and 95% confidence intervals for all intervals.

Shear Rate [s^{-1}]	**Test Fluids and Intervals**					
	dyn. Viscosity BCS [mPa·s]					
	0 Cycles		**1×10^3 Cycles**		**10×10^3 Cycles**	
	Mean	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**
10	1.122	[1.068; 1.176]	0.942	[0.789; 1.094]	0.919	[0.792; 1.045]
100	0.888	[0.739; 0.897]	0.891	[0.858; 0.923]	0.874	[0.823; 0.925]
1000	0.850	[0.835; 0.857]	0.891	[0.882; 0.899]	0.876	[0.869; 0.884]
	0.1×10^6 Cycles		**0.5×10^6 Cycles**		**1.0×10^6 Cycles**	
	Mean	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**
10	0.899	[0.783; 1.017]	0.951	[0.927; 0.974]	0.990	[0.966; 1.014]
100	0.846	[0.821; 0.871]	0.909	[0.904; 0.914]	0.946	[0.922; 0.969]
1000	0.859	[0.837; 0.880]	0.922	[0.912; 0.931]	0.965	[0.943; 0.986]
	dyn. Viscosity NBCS [mPa·s]					
	0 Cycles		**1×10^3 Cycles**		**10×10^3 Cycles**	
	Mean	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**
10	1.220	[1.146; 1.295]	0.875	[0.797; 0.953]	0.899	[0.826; 0.972]
100	0.912	[0.899; 0.925]	0.837	[0.824; 0.871]	0.848	[0.836; 0.880]
1000	0.863	[0.854; 0.872]	0.851	[0.834; 0.867]	0.862	[0.841; 0.883]
	0.1×10^6 Cycles		**0.5×10^6 Cycles**		**1.0×10^6 Cycles**	
	Mean	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**
10	0.862	[0.831; 0.893]	0.925	[0.887; 0.963]	0.931	[0.932; 0.943]
100	0.848	[0.838; 0.857]	0.898	[0.889; 0.908]	0.919	[0.913; 0.925]
1000	0.865	[0.852; 0.879]	0.915	[0.905; 0.926]	0.936	[0.931; 0.942]

In addition, the direct comparison of the two test sera in the test interval of 0 cycles (Figure 4c), 0.5×10^6 cycles (Figure 4d) and 1.0×10^6 cycles (Figure 4e), is shown. The results reveal that NBCS initially exhibits a higher dynamic viscosity than BCS (0 cycles). However, as soon as the sera are used in the test setup, the viscosity of NBCS is lower than that of BCS over all testing intervals for shear rates.

The statistical analysis of the progressions of the rheological examination over different test intervals showed normally distributed values for both BCS and NBCS for all measurements, as assessed by the Shapiro–Wilk test ($p > 0.05$). A repeated measures ANOVA with Greenhouse–Geisser correction showed a statistically significant difference between the test intervals of each sera (BCS: $F(1.433, 20.056) = 30.140$, $p < 0.001$; NBCS: $F(1.359, 25.822) = 23.653$, $p < 0.001$). A Bonferroni post hoc analysis between all intervals when tested with BCS revealed a significant difference at nearly all intervals compared to untested serum (0 cycles). Only at a test duration of 0.1×10^6 cycles, no significant difference was found ($p = 0.144$). A significant difference is observed for a test duration of 0.5×10^6 and 1.0×10^6 cycles compared to all others. When considering NBCS, the Bonferroni post hoc analysis revealed a significant difference when comparing test intervals to unused serum (0 cycles) for all intervals. Furthermore, the test intervals of 0.5×10^6 and 1.0×10^6 cycles also differ significantly from all others. Only the comparison between

0.5×10^6 and 1.0×10^6 cycles shows no significant difference ($p = 0.211$). The p-values of all comparisons can be found in Table 4.

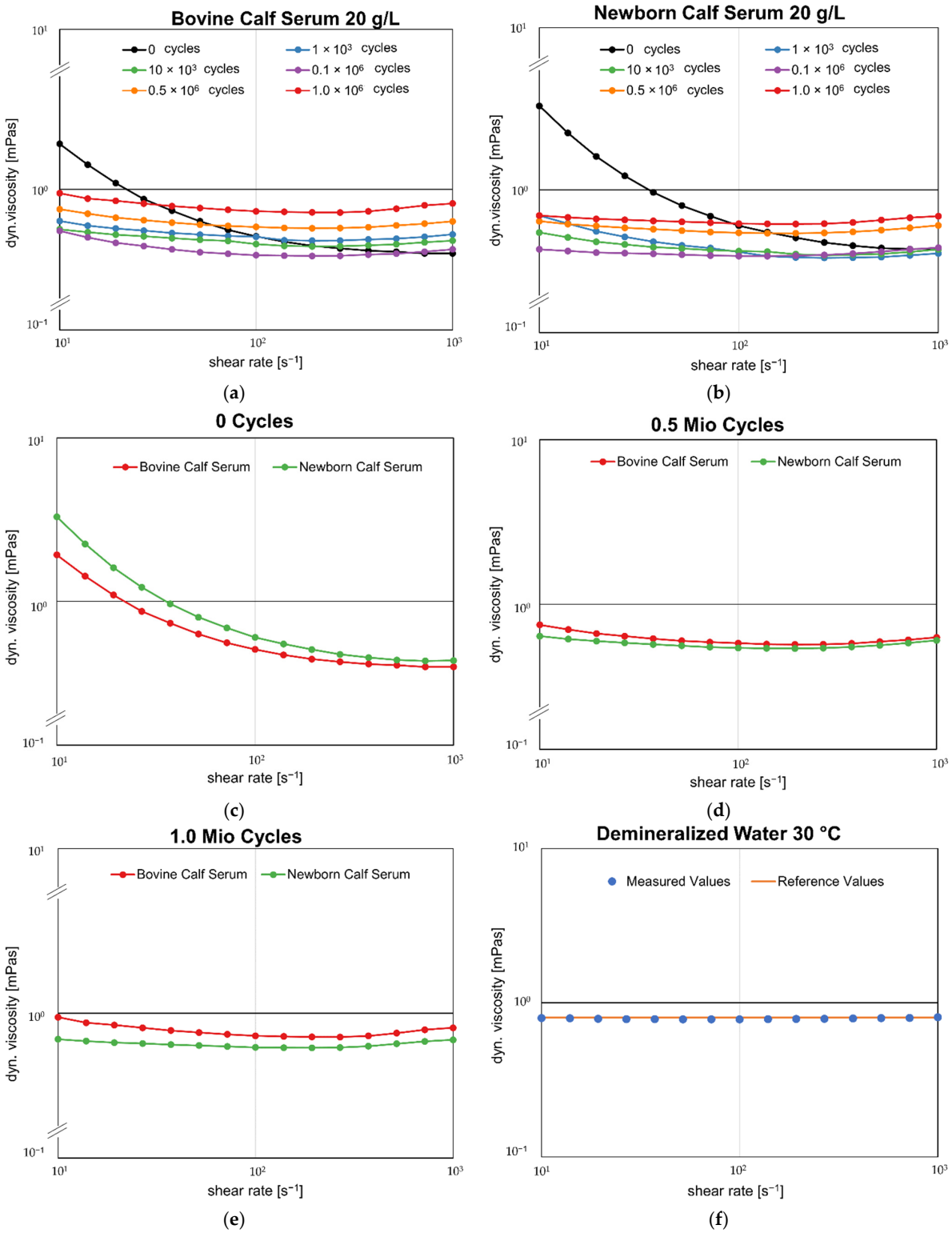


Figure 4. Comparison of the mean dynamic viscosity over shear rate: (**a**) bovine calf serum at all intervals; (**b**) newborn calf serum at all intervals; (**c**) both fluids—0 cycles; (**d**) both fluids—0.5×10^6 cycles; (**e**) both fluids—1.0×10^6 cycles; (**f**) comparison measurement of water at 30 °C and reference values ($\eta_{\text{water, 30°C}} = 0.8$ mPa·s).

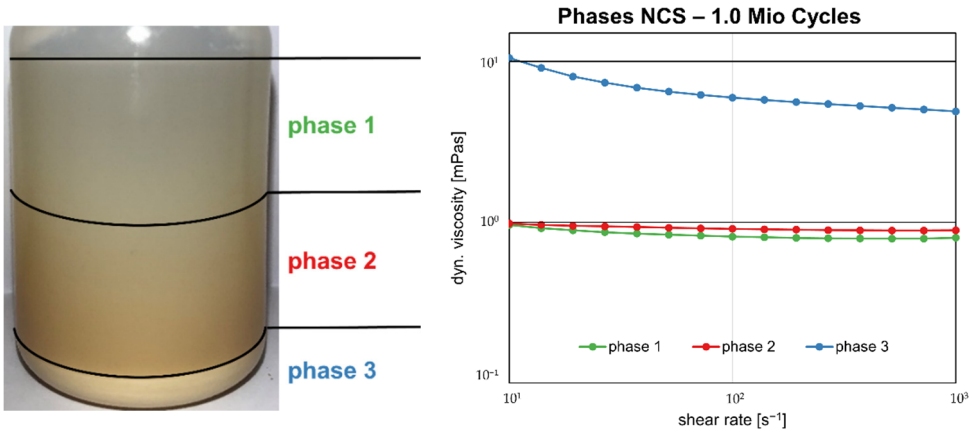
Table 4. Results of Bonferroni-adjusted post hoc analysis of the progressions of the rheological examination over different test intervals.

			BCS			
	0	**1×10^3**	**10×10^3**	**0.1×10^6**	**0.5×10^6**	**1.0×10^6**
0						
1×10^3	<0.001 *					
10×10^3	0.001 *	1.000				
0.1×10^6	0.144	0.014 *	0.390			
0.5×10^6	<0.001 *	0.045 *	0.003 *	<0.001 *		
1.0×10^6	<0.001 *	<0.001 *	<0.001 *	<0.001 *	0.005 *	
			NBCS			
	0	**1×10^{23}**	**10×10^{23}**	**0.1×10^6**	**0.5×10^6**	**1.0×10^6**
0						
1×10^3	<0.001 *					
10×10^3	0.025 *	1.000				
0.1×10^6	0.020 *	1.000	1.000			
0.5×10^6	0.019 *	<0.001 *	<0.001 *	<0.001 *		
1.0×10^6	<0.001 *	<0.001 *	<0.001 *	<0.001 *	0.211	

* = Statistically significant.

In Figure 4f, the averaged measured viscosity of deionized water at 30 °C at different shear rates is shown. The deviation of measured viscosity from the theoretical reference value of water is less than 2.6% over the entire shear rate range. The rheological results with deionized water at 30 °C also showed a normal distribution.

Both sera formed three optical distinguishable phases after the serum was placed in a temperature-controlled measurement room for 24 h. These phases are exemplified by the NBCS with run time of 1.0×10^6 cycles (see Figure 5a). Here, a clear almost transparent phase (phase 1) can be identified at the top, a turbid phase (phase 3) at the bottom and a mixed phase (phase 2) in the middle. Figure 5b illustrates the results of the measured dynamic viscosity over increasing shear rates for the three evoked distinguishable phases of NBCS. This shows an increasing viscosity from phase 1 to phase 3 over the complete shear rate range. Especially, the rheological curve of the third phase shows a continuously decreasing dynamic viscosity with increasing shear rate. In addition, phase 1 shows a more pronounced shear-thinning behavior than phase 2.



(**a**) (**b**)

Figure 5. (**a**) Three optically distinguishable phases of the newborn calf serum; (**b**) comparison of the mean dynamic viscosity over shear rate for the three evoked distinguishable phases of newborn calf serum.

To ensure laminar flow, the Reynold numbers were calculated in the annular gaps at the maximum shear rate of $\dot{\gamma} = 1000\ s^{-1}$ for both sera at all intervals. With $R_e = 13.79$ mm and $R_i = 13.31$ mm, an annular gap of $L = 0.48$ mm results. Using the rotational speed from the measured values of the rheometer, a maximum velocity referring to the gap center of $v = 0.474\ \frac{m}{s}$ was obtained. With the respective densities of the two sera, the Reynold numbers are in the range of $Re = 237 - 261$ for NBCS and $Re = 230 - 271$ for BCS. The critical Reynolds number at which turbulent flows are expected in the annular gab of double gap measuring systems is $Re_{crit} \geq 1000$.

3.4. Protein Concentration

Table 5 shows the measurement results of the protein concentrations before testing and of the three forming phases exemplified by NBCS after 1.0×10^6 cycles. Before starting the test, the protein concentration was within the 20 ± 1 g/L for both test sera as described in ISO 14243-1 for both test sera. The protein concentrations of the three phases after 1.0×10^6 cycles differ clearly from each other, with the middle phase (phase 2) almost corresponding to the unloaded serum, the upper phase (phase 1) having a very low protein concentration, and the lower phase (phase 3) having a considerably increased concentration of proteins.

Table 5. Measurement results of protein concentrations before testing and the phases after 1.0×10^6 cycles.

	Protein Concentration NBCS [g/L]			
	0 Cycles	**1.0×10^6 Cycles**		
		Phase 1	**Phase 2**	**Phase 3**
MV ± SD	19.98 ± 0.07	2.63 ± 0.12	16.30 ± 0.72	55.27 ± 10.13

4. Discussion

Due to the imprecise definition of the test medium to be used in in vitro wear tests, this study outlined the direct comparison of two possible test fluids. BCS and NBCS, both suitable according to ISO standards, were investigated with respect to their rheological properties. In addition, the change in rheological properties during a biomechanical test was considered Previous studies had already demonstrated the suitability of BCS in terms of rheological properties for biomechanical examinations. In addition, a dependence of dynamic viscosity on shear rate, temperature, pressure, and protein concentration was demonstrated [26,27]. Nevertheless, little is known about the rheological behavior of NBCS, although it is used just as frequently as BCS in biomechanical examinations. Furthermore, a change of rheological properties due to biomechanical testing has not yet been taken into account for either of the sera.

4.1. Do NBCS and BCS Show Different Rheological Properties?

Comparing the rheological behavior of BCS and NBCS based on the results of the rheometric analysis before testing (0 cycles), both sera exhibited shear thinning behavior in the range of low shear rates ($\gamma \leq 100\ s^{-1}$) and nearly Newtonian behavior in the range of high shear rates ($\gamma > 100\ s^{-1}$). In addition, the results revealed that NBCS initially shows a higher dynamic viscosity than BCS (0 cycles) and at higher testing intervals the viscosity of NBCS is lower than that of BCS over the entire shear rate range. Nevertheless, this difference between the sera was not statistically significant, allowing the conclusion that BSC and NBCS do not differ from each other in terms of their rheological behavior before (0 cycles) and after biomechanical examinations (0.5×10^6 cycles).

There are few studies investigating the influence of different components of serum as test fluid. Bortel et al. report a constant dynamic viscosity of 0.94 ± 0.03 mPa·s over the shear rate range of $\gamma = 1 - 1000\ s^{-1}$ in the rheological study of NBCS with a protein concentration of 30 g/L [33]. In addition to the higher protein concentration, the amount of additives incorporated into the serum differed from those in this study. Bortel et al.

added 2 g/L sodium azide and 3 g/L EDTA. The influence of this variation of additives on the rheological behavior has not yet been investigated, but it is known that a different biochemical composition of the test fluid has an influence on the polyethylene wear [34]. Furthermore, it was shown that the composition of the serum can additionally have an influence on the fluid film thickness between the sliding surfaces. Especially the amount and composition of the albumins and globulins should play a decisive role in this context [35]. However, the present rheological study shows no statistically significant influence of the different composition of the sera on the rheological properties. The effect of different compositions on the wear behavior in biomechanical examinations has to be verified in further investigations. As at least six measuring points are required as standard for the determination of wear rate, this could not be obtained within this study, since only a single averaged measured vale from the three wear stations per interval could be collected. Moreover, no reference control was included in this test.

4.2. Do the Rheological Properties of the Two Fluids Change after Different Testing Intervals?

When the two sera were analyzed at different intervals during the biomechanical investigation, a clear variation in rheological behavior was seen for BCS at shear rates $\gamma > 100\ s^{-1}$. This variation was not evident with NBCS. Nevertheless, for both sera, the rheological behavior change was statistically significant as soon as the test started. In addition, a significant change in the rheology of both sera was obvious between intervals greater than 0.1×10^6 cycles. Only NBCS showed no significant change from the interval of 0.5×10^6 to 1.0×10^6 cycles. These results verified that the rheological behavior of test sera changed as soon as they were mechanically loaded. This implies that the replacement interval of the serum plays a decisive role, as the fluid exhibits a more viscous behavior with increasing test duration and thus influences the wear behavior in biomechanical examinations. This correlation has already been demonstrated by Reinders et al. in a knee-wear simulator test. In this, it was confirmed that a reduction in replacement interval leads to an increase in wear rate of polyethylene inserts [28]. From this context, it can be assumed that wear decreases towards the end of the normative interval, as the viscosity of the fluid increases.

This effect can be explained by the degradation of serum proteins. Due to frictional heating generated at the articulating interface during testing, the constituents in serum decompose over time [36,37]. In addition, proteins and other components of the serum can be denatured by locally increasing temperature and direct pressure [38,39]. The decomposed products are absent in vivo due to continuous regeneration of proteins. In vitro, however, these products remain as precipitates and may influence the wear behavior as a solid lubricant by deposition between articulation surfaces [36,40,41]. The change in serum and the presence of precipitation products can be demonstrated by segmentation of the heavier degraded and denatured proteins, as shown in Figure 5a. Here, after 24 h of storing the test fluid by room temperature, three evoked distinguishable phases appear. An increasing viscosity from phase 1 to phase 3 over the complete shear rate range can be detected. Accordingly, this rheological analysis supports the statement that the precipitation products can lead to a minimization of wear.

Varying prosthesis designs in combination with the congruency of the sliding partners and their alignment can lead to a major change in contact pressure [42,43]. In addition to the design, the considered body weight and the level of daily activity also plays a decisive role [13]. Whether and to what extent these changes in contact pressure between the sliding surfaces have an influence on the rheological behavior of the test fluid during a tribological test has not yet been examined in detail. Therefore, further studies should investigate whether the rheological properties of the fluids could change over the test duration, depending on contact pressure.

Another change in serum during the biomechanical test that may affect wear behavior is the change in protein concentration. It has already been shown that an increase in protein concentration leads to an increase in dynamic viscosity [26]. In addition, the wear rate is

reduced by increasing the protein content of the serum [36,41]. Considering the protein concentration determined in the three forming phases after the test, there is an increased protein concentration in the bottom depositing phase (phase 3). In addition, the standards specify that the fluid lost by evaporation during the test must be replaced at least daily by adding deionized water. This can lead to a dilution of the test medium and a change in protein concentration, as the precise amount of evaporated water is typically unknown. Furthermore, sufficient mixing of the existing serum and the added deionized water cannot be guaranteed. This procedure causes that a comparability between different test setups is not ensured since the evaporation of the test fluid is mainly influenced by the setup of the test chamber. This may additionally lead to a change in the wear behavior during biomechanical examinations. In this study, sealed chambers were used to minimize the leakage of evaporated water.

4.3. Limitations

Some attention should also be given to the quality and repeatability of the measurements. In the results, an increased standard deviation can be seen at small shear rates, especially in the measurements of the dynamic viscosity of the sera before testing. This standard deviation decreases after mechanical loading of the sera. This effect could be attributed to the sedimentation of proteins in the fluid. The rate of sedimentation depends on the density and size of proteins and the density of the tested sera. The size of the proteins, their composition and accordingly their density may change due to denaturation and degradation under mechanical stress. The lower standard deviation in the rheological examination of the loaded sera could therefore be explained by the fact that a more homogeneous distribution of the proteins may have been established by the mechanical loading. The exact processes during sedimentation and the differing rates of the various proteins should be considered in more detail in further studies. It should also be mentioned that the process to provoke a segmentation of the severely degraded and denatured proteins was carried out at room temperature (21 °C) not at 37 °C as during the in vitro simulation. It is assumed that the temperature did not affect the result of the segmentation in the three optical distinguishable phases, and only the speed of the process may have been influenced.

To validate the test method, a reference measurement of deionized water was performed. The deviation of measured viscosity of deionized water at 30 °C from the theoretical reference value of water is less than 2.6% over the entire shear rate range. In addition, the technical data of the used rheometer has already been discussed and proven to be capable [26]. The highest Reynold number with $Re = 271$ was determined for BCS before the mechanical loading started. Since this is well below the critical Reynolds number $Re_{crit} \geq 1000$, turbulent flows should not occur during the measurement. This allows the assumption of the validity of the measurement method.

It should be mentioned lastly that the focus of this study was on the physical properties of the test fluid. Besides the rheological properties, other factors may have a decisive role in evaluation of the medium for wear tests. The influence of biochemical factors such as osmolarity and thermal stability of the components [34,44] and the chemical effect of molecular compounds on the wear behavior should not be neglected [45]. Furthermore, in the context of this study, the changes of the protein structure were considered to be the main cause for the changing rheological behavior of the calf sera. In addition, the phospholipids can also have an influence on this behavior [36]. That phospholipids have an influence on polyethylene wear has already been demonstrated [46,47]. However, the percentage of lipids compared to the proteins is very low, which is why lipids content in calf serum is assumed to be negligible. The precise influence of lipids on the rheological behavior and changes of calf sera should be conclusively considered in further studies.

5. Conclusions

The current study has explored the rheological behavior of newborn calf serum and bovine calf serum, which are approved according to ISO 14242 and ISO 14243 for wear

testing of endoprosthetic components. In particular, the suitability of the sera for biomechanical testing have been considered, whereby the change of the properties during the test was of special interest. From the research conducted, the initial questions can be answered as follows:

1. BSC and NBCS do not differ from each other in terms of their rheological behavior neither before (0 cycles) nor after biomechanical examinations (0.5×10^6 cycles).
2. The rheological behavior of test sera changes as soon as they are mechanically loaded. In addition, the replacement interval of the serum plays a decisive role in biomechanical testing, as the fluid exhibits a more viscous behavior with increasing test duration.

These findings, each alone or all together, could influence the wear behavior during biomechanical examinations. The extent to which the individual factors could affect the wear rates determined in wear tests should be considered in further investigations.

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