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The Effects of Lactic Acid on PGE_2 Production by Macrophages and Human Synovial Fibroblasts: a Possible Explanation for Problems Associated with the Degradation of Poly(lactide) Implants?

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Abstract: The aim of this study was to investigate the effects of lactic acid on cells found at the bone–implant interface in order to try to discover more about the effects of degradation of implants manufactured from poly(lactide). This study shows that human synovial fibroblasts and murine macrophages release prostaglandin E_2 (PGE_2) a bone resorbing and inflammatory mediator, into the surrounding medium when exposed to lactic acid. The production of PGE_2 in response to lactate may help to explain isolated cases of inflammation and discomfort seen some time after fracture fixation with poly(lactide) implants.

INTRODUCTION

Poly(lactide) implants have been used in animal studies and clinical trials as bone implants for fixation of a variety of fractures.[1] Whilst in the main fracture fixation has been successful, a few patients have experienced inflammation and swelling in the vicinity of the implants.[2] Often the presence of poly(lactide) particulates has been associated with these problems and therefore the degradation of the material may be responsible. The poly(lactide) ultimately degrades into the lactate monomer and it was initially thought that since lactate is converted into carbon dioxide and water by the tricarboxylic acid cycle in the body, it would be unlikely that degradation of the polymer would result in any biocompatibility problems.

The aim of this study was to investigate the effect of lactate, by exposing cells likely to be present at the bone–implant interface to a range of concentrations. Cell viability and number were assessed by morphological studies and lactate dehydrogenase (LDH) assays. Release of LDH into the medium provided an indication of cell membrane damage. Levels of the inflammatory bone resorbing mediator PGE_2 were also measured.

MATERIALS AND METHODS

Cell types

Macrophages and fibroblasts were studied since these are cells which can reside for a long time in the vicinity of an implant. Murine macrophages of the cell line IC21 were obtained from the European Collection of Animal Cell Cultures (ECACC), Porton Down, UK. Cells were cultured in 150 cm^2 tissue culture flasks using RPMI 1640 cell culture medium containing 10% (v/v) foetal calf serum, 1% (v/v) glutamine and 1% (v/v) penicillin–streptomycin. Cells were allowed to reach a level of 80% confluency before they were washed with phosphate buffered saline (PBS), (without calcium and magnesium) and split using a non enzymatic cell dissociation solution prior to being seeded into test wells.

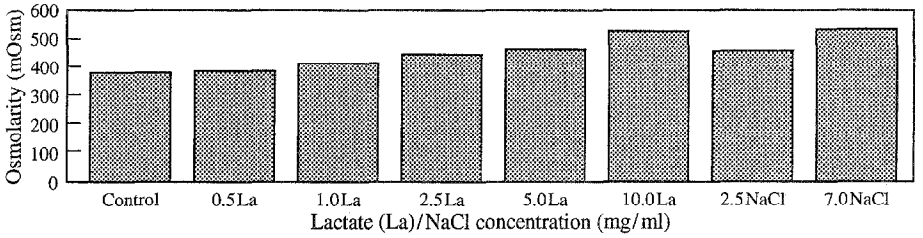

Fig. 1. Osmolarity changes of DMEM cell culture medium (supplemented with 10% FCS, 1% glutamine and 1% pen–strep), when lactate or NaCl are added.

For the culture of normal human synovial fibroblasts a small biopsy of synovium was taken during arthroscopic examination of the knee. The tissue was maintained under aseptic conditions and cells cultured according to the methods of Rae (1981).[3] The cells were subcultured in 150 cm^2 tissue culture flasks using DMEM supplemented with 10% foetal calf serum, 1% (v/v) glutamine and 1% (v/v) penicillin–streptomycin, to a level of 70–80% confluency and then washed using PBS (without calcium and magnesium) and split with trypsin-EDTA.

Both types of cells were grown at 37 °C in an atmosphere of 5% CO_2 in air.

Test media preparation

The cell culture media as described in the above sections were used as negative controls. Media containing lactate were made by serial dilution of cell culture medium containing 10 mg/ml lactic acid (L (+) lactic acid 98% purity from Sigma). The 10 mg/ml lactic acid stock was pH adjusted to pH 7·4 (pH of normal culture medium), using 1M NaOH, this meant that the effect of pH was eliminated as one of the test variables. The osmolarity changes associated with the addition of lactic acid were controlled for by preparing media containing known quantities of NaCl such that the NaCl containing media had similar osmolarities to the lactate solutions; any differences seen between the NaCl and lactate test wells would thus be due to the presence of these molecules alone.[4] Osmolarity was measured using a Camlab osmometer. The osmolarities of all test media are shown in Figs 1 and 2. All media were filter sterilised prior to use.

In vitro test method

Three identical experiments were carried out for each cell type. Cells were seeded into 24 welled plates containing sterile coverslips for morphology studies, or into 96 welled plates for test wells. Macrophages were seeded out so that there were approximately 50,000 cells per cm^2 and fibroblasts so that there were 25,000 cells per cm^2. Twenty four hours after seeding the cells out, the cell culture medium was aspirated from each well and replaced with the appropriate test medium; 140 μL per well for test wells or 1 ml for morphology wells. Four replicate test wells were set up for each test medium and time point; two for LDH and two for PGE_2 assays. At intervals of 24, 48 and 72 h after the addition of test media, media samples were removed from test wells and aliquoted into Eppendorf tubes. Media samples were refrigerated for LDH analysis the following day or frozen at −70°C for later PGE_2 analysis. One hundred and forty microlitres of 1% triton in PBS was then added to the test wells and these were refrigerated for 24 h so that any remaining cells would lyse. The resultant cell lysates were then aliquoted

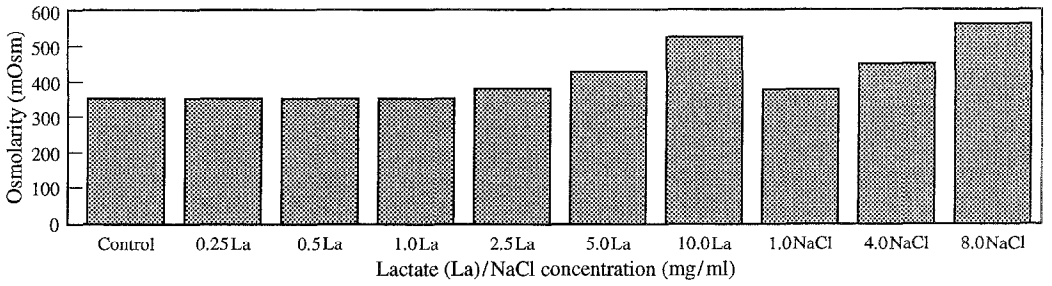

Fig. 2. Osmolarity changes of RPMI cell culture medium (supplemented with 10% FCS, 1% glutamine and 1% pen–strep), when lactate or NaCl are added.

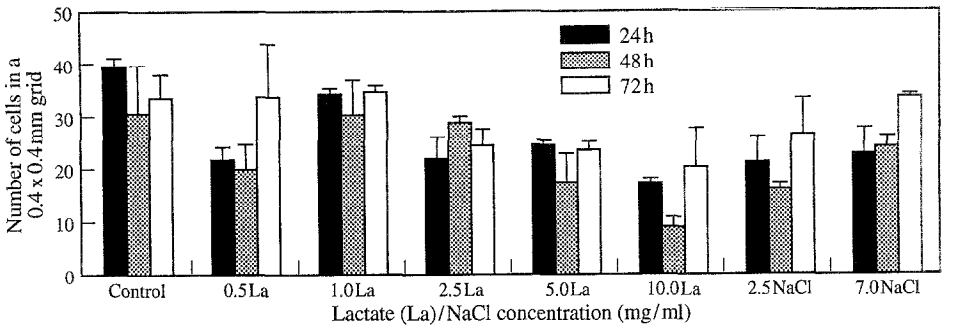

Fig. 3. Human synovial fibroblast cell number results as determined by light microscopy (100 × mag) of coverslips from morphology wells.

into eppendorf tubes and used in LDH assays. Media were removed from morphology wells at the same intervals and replaced with 1 ml of methanol per well for fixation. Cover slips were then stained with May Grunwald and Giemsa and mounted on to microscope slides with DPX.

LDH assay

The LDH activity in 50 μL aliquots of each sample was assessed using the CytoTOX 96^{TM} (Promega) non radioactive cytotoxicity assay.[5] Absorbance values (abs) were recorded in a 96 well format with a standard ELISA plate reader at 492 nm. The percentage of LDH released from cells into the medium was taken as a measure of cell membrane integrity. The percentage of LDH released per test well was expressed as follows:

$$\% \text{ LDH release} = [(\text{abs of medium})/(\text{abs of medium} + \text{abs of lysate})] \times 100$$

The LDH values obtained from the cell lysates alone were also examined in order to establish whether an increased external lactate concentration affected the production of the enzyme by the cell.

PGE_2 assay

PGE_2 in medium samples was assayed using the BIOTRAK Prostaglandin E_2 (Amersham Life Science), enzyme immunoassay (EIA) system.

Morphology

Coverslips were examined using a light microscope. The number of cells in a standard area were counted in order to obtain an estimate of cell viability.

Statistics

Paired two tailed *t*-tests were carried out in order to compare results from different test wells. Values of $P(T <= t)$ are presented. The null hypothesis was that there was no difference between groups and P was taken to show significant differences between groups if less than or equal to 0·05.

RESULTS

Cell numbers as determined by morphology studies

Fibroblasts

At a concentration of 0·5 mg/ml lactate a decline in cell numbers could be noted at 24 h. Despite this cells appeared to be confluent and one could postulate that the remaining cells had either become larger or more spread out. By 72 h with 0·5 mg/ml lactate however cell numbers had increased so that they were again equivalent to controls. With concentrations of 2·5 mg/ml and 5 mg/ml lactate cells appeared to be healthy but were beginning to cluster together. A 10 mg/ml concentration of lactate was very toxic and often only a few isolated cells with shrunken cytoplasms remained. At both NaCl concentrations cells were confluent at all time points however at 24 h there were fewer cells per unit area in NaCl test wells than in the negative control wells but by 72 h equivalent numbers of cells were present.

Macrophages

Increasing lactate concentration resulted in the elongation of lamellipodia at 2·5 mg/ml lactate and eventual cell death at 10 mg/ml lactate. NaCl

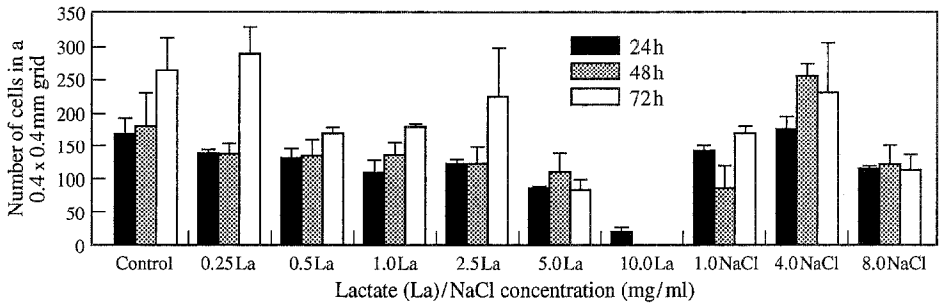

Fig. 4. Murine macrophage cell numbers as determined by light microscopy (100 × mag) of coverslips from morphology wells.

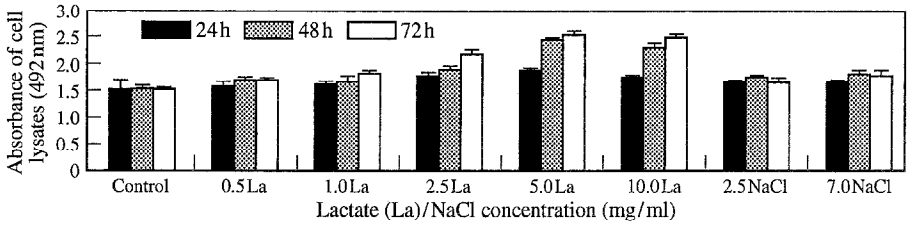

Fig. 5. Human synovial fibroblast cell lysate LDH absorbance values. (The means and standard errors of three replicate experiments are shown.)

solutions of 1 and 4 mg/ml did not appear to have any effect on cell numbers although some cells did have extended lamellipodia. With 8 mg/ml NaCl cytoplasms were not that well stained at 72 h and cell numbers were lower than in negative control wells at all time points.

Cell lysate LDH absorbance values

Fibroblasts

The presence of the lactate in the medium at levels of 2·5 mg/ml and above, promoted a much greater production of lactate dehydrogenase in the remaining living cells; this was found to be statistically different from controls at the 48 and 72 h time points, (2·5 mg/ml lactate: 48 h $P = 0{\cdot}011$, 72 h $P = 0{\cdot}003$; 5 mg/ml lactate: 48 h $P = 0{\cdot}005$, 72h $P = 0{\cdot}007$; 10 mg/ml lactate: 48h $P = 0{\cdot}002$, 72 h $P = 0{\cdot}004$). NaCl solutions caused a less dramatic but nevertheless significant increase in LDH production in comparison to controls (2.5 mg/ml NaCl: 48 h $P = 0{\cdot}024$, 72 h $P = 0{\cdot}038$; 7 mg/ml NaCl: 48 h $P = 0{\cdot}027$, 72 h $P = 0{\cdot}049$). Lactate dehydrogenase converts excessive levels of lactate entering the cells into pyruvate and NADH.

Macrophages

At 5 and 10 mg/ml lactate and 8 mg/ml NaCl, the cell lysate LDH absorbance values were lower than those of cells in negative control wells at all time points. These results corresponded with the morphology results and thus reflected cell viability changes as demonstrated by comparing Fig. 6 to Fig. 4.

% LDH release into the medium

Fibroblasts

It would appear that there was very little difference between the LDH release of negative controls and NaCl osmolarity controls and the *P*-values of *t*-tests at all time points confirmed this. In lactate

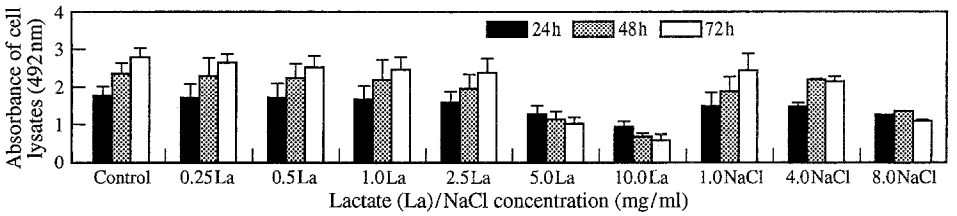

Fig. 6. Murine macrophage cell lysate LDH absorbance values. (The means and standard errors of three replicate experiments are shown.)

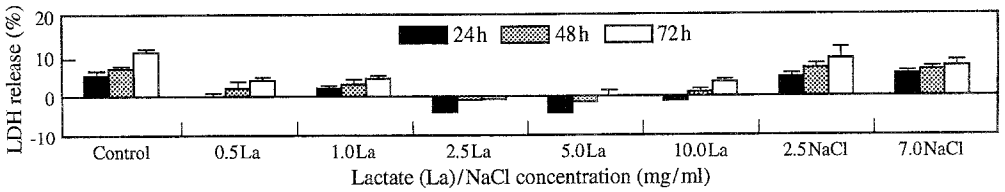

Fig. 7. % LDH release into the medium by human synovial fibroblasts. (The means and standard errors of three replicate experiments are shown.)

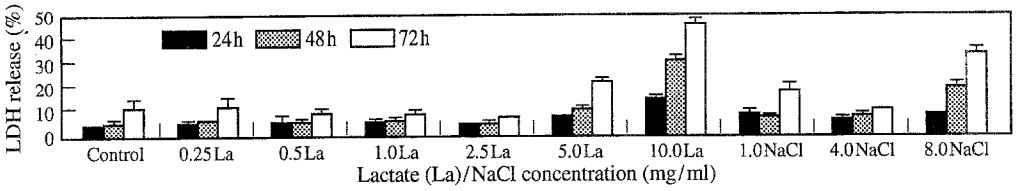

Fig. 8. % LDH release into the medium by murine macrophages. (The means and standard errors of three replicate experiments are shown.)

test wells however percentage LDH release was depressed and negative results were obtained when the absorbance readings fell below the 'back ground' absorbances of normal culture medium which had not been exposed to cells. The reason for the negative results may have been due to the presence of the lactate in the medium; possibly any LDH released from cells into the medium in its active form became inactive once it had reacted with the lactate. It is also possible that other enzymes released by dying cells destroyed any LDH in the medium.

Macrophages

With macrophages it would appear that the presence of lactate did not have any effect on LDH release in comparison to negative controls until a concentration of 5 mg/ml was reached (24 h $P = 0{\cdot}02$, 48 h $P = 0{\cdot}003$, 72 h $P = 0{\cdot}013$), after which percentage LDH release increased dramatically. This corresponded with the cell number changes and was obviously linked to cell death and subsequent release of cytoplasmic LDH into the medium. The presence of NaCl at a concentration of 8 mg/ml increased LDH release although it did not increase it to the same extent as 10 mg/ml lactate. Comparison of 10 mg/ml lactate to 8 mg/ml NaCl results did not however reveal any differences that were statistically significant. (n.b. 8 mg/ml NaCl and 10 mg/ml lactate solutions had similar osmolarities).

PGE_2 release

Fibroblasts

The presence of concentrations of lactate as low as 0·5 mg/ml produced an increase in PGE_2 release into the medium at 24 h when compared to controls, ($P = 0{\cdot}008$). Concentrations of PGE_2 released did not appear to increase in a dose dependent manner after 1 mg/ml lactate was reached. Exposure of fibroblasts to NaCl did not cause such an increase in PGE_2 release. This is especially obvious if 2.5 mg/ml NaCl test wells are compared with 5 mg/ml lactate test wells, ($P = 0{\cdot}003$) one should note that both test solutions had similar

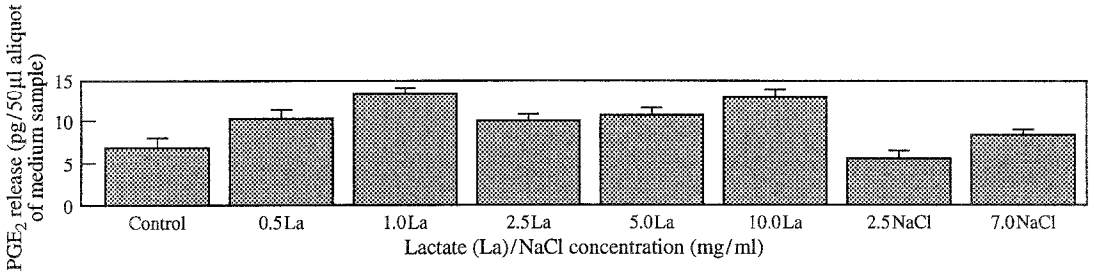

Fig. 9. PGE_2 release from human synovial fibroblasts when exposed to lactate and NaCl for 24 h. (The means and standard errors of three replicate experiments are shown).

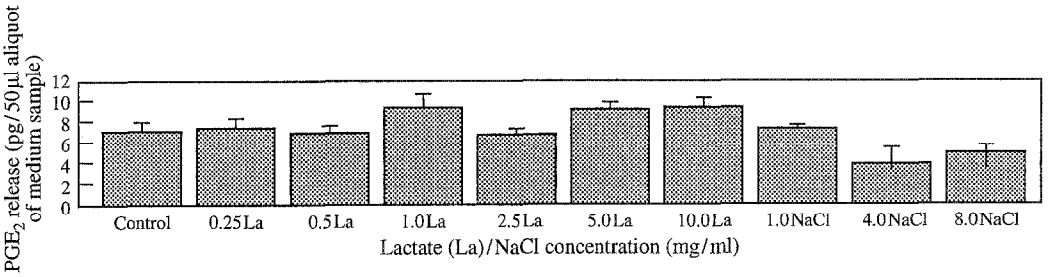

Fig. 10. PGE_2 release from murine macrophages when exposed to lactate and NaCl for 24 h. (The means and standard errors of three replicate experiments are shown.)

osmolarities and cell numbers at 24 h were similar.

Macrophages

PGE_2 release into the medium was increased although not with statistical significance, with 1·0 and 5 mg/ml of lactate at 24 h. At 10 mg/ml lactate PGE_2 release was significantly greater than in control wells, ($P = 0{\cdot}004$). At 1 mg/ml lactate, increased release was unlikely to be due to cell lysis however at 5 and 10 mg/ml, lysis may well have been a contributing factor. NaCl appeared to reduce PGE_2 production by macrophages although this reduction was not statistically significant.

DISCUSSION

As demonstrated by comparing the effects of lactate solutions with the effects of NaCl solutions of similar osmolarities, the results seem to indicate that the presence of the lactate molecule itself and not just the associated osmolarity changes affected the cells in these experiments. Some very similar experiments investigating cell proliferation in the presence of lactate have been carried out by other authors[4,6] however, they used different cell types and different time points to ours making it difficult to compare results accurately. Despite this a similar conclusion was drawn: that decreased rates of cell proliferation in the presence of lactate were likely to be due to the presence of the monomer itself and not just to the osmolarity changes.

Fibroblasts seemingly became larger in volume at 0.5 mg/ml lactate and at higher concentrations they began to form clusters. Macrophages on the other hand did not show any major morphological changes until 2·5 mg/ml lactate was reached and at this point elongation of lamellipodia was noted. Ten mg/ml lactate was toxic to both cell types.

The presence of lactate can cause both macrophage and fibroblast cell death; however both cell types behaved differently in terms of their metabolic response to lactate. This is exemplified by the increase in internal LDH production by fibroblasts compared to the release of internal LDH by macrophages. The fibroblast 'deals' with the new extracellular environment by converting the extra lactate entering the cell into pyruvate whereas the macrophage merely bursts and releases any LDH it contains directly into the medium. This is not surprising when considering the roles of these two cells in the body; the macrophage is there to help protect and clean the environment in which other important cells live and thereby by bursting and releasing LDH in order to remove lactate it may be fulfilling this role. The fibroblast on the other hand is an important component of connective tissue and must therefore remain intact thus by increasing its internal LDH levels so that it can keep the lactate levels within its cytoplasm as constant as possible it may achieve this goal. Incidentally the increased levels of cytoplasmic LDH activity may partly explain the larger cytoplasmic volume of fibroblast cells exposed to lactate.

Both cell types released increased levels of PGE_2 into the medium in response to being exposed to lactate with fibroblasts being more sensitive and increasing PGE_2 production at concentrations as low as 0·5 mg/ml lactate. These slight increases in PGE_2 release may help to explain the rare clinical cases of intermittent swellings and discomfort associated with degrading poly(lactide) implants[2–9] however one should bear in mind that other inflammatory mediators may also be involved.

It is interesting to note that many studies have been conducted in the past on the increased lactate levels seen in the synovial fluids of arthritic patients;[10–15] it would be very interesting on the basis of these results to test to see whether these

increased levels of lactate in the synovial fluids of arthritic patients are also associated with increased PGE_2 levels. If high lactate levels can initiate PGE_2 production then it is possible that the final stages in degradation of a poly(lactide) implant could result in inflammation and bone resorption. So far clinical cases of osteolysis have only been associated with poly(glycolide) implants however, bearing in mind that these degrade at a faster rate than poly(lactide) implants, it is possible that no human clinical studies have yet run for long enough for similar problems to come to light with poly(lactide). Some recent animal studies using dogs[16] have however shown regions of bone resorption associated with poly(lactide) implants.

The main limitation of this study is its *in vitro* nature and the fact that the macrophages are not of human origin. Nevertheless the murine macrophage system of biocompatibility testing is a well established one. The effects of pH changes associated with lactate concentration were not investigated. The 10 mg/ml lactate solutions would undoubtedly have caused cell death had they not been buffered and it is unknown as to whether such high lactate concentrations would be realistically found in tissues surrounding degrading poly(lactide) implants. Future studies looking at the effects of slight changes in pH associated with lactate accumulation would however definitely be worth while especially since acid reactions of pH 3–4 have been found in inflamed tissues surrounding degrading Biofix (poly(glycolide)) bone implants.[17] Sodium chloride was chosen as an osmolarity control in order to compare our findings with those of other authors[4,6] however it could justifiably be argued that other molecules such as sucrose would be equally appropriate. Sodium hydroxide was added (as in experiments by previous authors[4,6]) to adjust the pH of the lactate solutions however alternative buffering systems such as HEPES could also have been used.

In conclusion, the presence of lactate appears to cause alterations in cell mortality, percentage LDH release and increases in PGE_2 release which are not attributable to osmolarity changes alone. The PGE_2 release may help to explain the inflammation seen in some patients however until samples drained from swellings associated with poly(lactide) implants are tested for lactate and this inflammatory mediator, one cannot be absolutely certain.

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