INTRODUCTION:
Clinical application of in vitro expanded multipotent mesenchymal stromal cells (mesenchymal stem cells, MSC) is subject to specified requirements and regulations. Thus, it is reasonable to use autologous tissues and cells as much as possible in order to avoid possible allo- or xenograft-induced immune reactions or the transfection of pathogens. The fixation of expanded MSC using freshly prepared autologous plasma will cover these clinical requirements. By varying the calcium concentration, plasma clots with different properties can be produced.

The purpose of this in vitro study was to determine an optimal calcium concentration for the clotting process, clot stability and intra-clot cell viability for stem cell delivery in orthopedic surgery.

METHODS:
Platelet-containing plasma was prepared by centrifugation (700xg, 8min) of citrated peripheral blood. Plasma clots were performed by adding an equal volume of RPMI1640 (with/ without hMSC) and 10% CaCl₂ to the plasma. The final concentration of CaCl₂ ranged from 1% to 10% (v/v) of plasma. After mixing, plasma was allowed to clot up to 90 min in a cell incubator.

Clotting time was recorded when a solid clot formation was apparent. Clot morphology was evaluated and photographed after incubation within RPMI1640 under cell culture conditions up to 3 weeks. Viability and distribution of MSC was analyzed by calcein-AM/propidium iodide staining up to 3 weeks. MSC-embedded plasma clots were dissolved with trypsin (0.25%) and recovered cells were incubated for 1 week under cell culture conditions. Subsequently, MSC were stained (Pappenheim staining) and evaluated by light microscopy.

A clot rupture model was developed and used for quantitative rupture force analysis of the different clots. Using 12-well cell culture plates, a disc (diameter: 16 mm) was dissected from the middle of each well and the hole was subsequently closed by a sealing film (Fig 1A). Different plasma clot mixtures were prepared as described.

RESULTS:
Variation of calcium concentration affected clotting time. At CaCl₂ concentration between 1% and 7% 20-30 min was recorded until clot formation was apparent whereas concentrations of 8%-10% CaCl₂ caused an increase in clotting time (60 min).

Additionally, the different calcium concentrations yielded in plasma clots with different appearance (transparent gelatinous formations, opaque with high contraction or partial liquid). As shown in Fig 2, plasma clots, composed with 1%-10% CaCl₂ showed no further change in shape and consistence after storage for 3 weeks within a cell incubator within pure RPMI1640.

Fig 2: Clot appearance and consistence in RPMI1640 for 3 weeks.

The viability of embedded MSC was not affected up to 3 weeks when clots were polymerized with CaCl₂ concentrations of 1% up to 5% (Fig 2A), whereas clots containing 6%-10% showed a decreased number of viable cells and an increased number of dead cells (Fig 2B).

Fig 2: Cell viability after 1 week within plasma clots composed with A: 5% and B: 10% CaCl₂. Calcein-AM/propidium iodide staining discriminates live cells (green) and dead cells (red) (magnification x5).

Traspin efficiently lysed all clots within 50 min under cell culture conditions. Dissolution times of clots, composed with 1%-4% CaCl₂ were 16% up to 50% and 60% shorter than those of clots with 5% or 6%-8% or 9-10% CaCl₂.

Cell morphological analysis of stained recovered MSC revealed that this lysis of the clots did not affect cell morphology or a subsequent spontaneous proliferation. However, the cell recovery from low calcium clots (1%-5%) was significantly higher compared to high calcium (6%-10%) (Fig 3 A).

Fig 3: Cell recovery of clots containing 5% CaCl₂ or 10% CaCl₂. Pappenheim staining (magnification: x5).

Quantitative rupture analysis demonstrated, that the stabilities of clots containing 1% up to 4% CaCl₂ were approximately the same for all samples (resistance force 0.229N-0.240N), while the stabilities of clots containing 5% up to 10% CaCl₂ were constantly lower (0.151N-0.179N). The presence of MSC within clot matrices (1%-5% CaCl₂) resulted in an increase in the stability of each clot (0.244N-0.293N).

DISCUSSION:
Clotting time, clot formation and stability can be controlled by changing CaCl₂ concentration. Clots containing 5% CaCl₂ produce optimal results for stem cell delivery. Here, the clotting process is relatively short (up to 25 min), consequently excessive waiting time during surgery can be avoided. Immediately after gelation these clots appear transparent but elastic and stable and can be easily handled probably to fill up a bone defect.