

Reducing the Invasiveness of Obtaining Blood-borne Measures in Animals

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Summary — To overcome the limitations associated with routine blood collection methods in welfare research, we are developing a new technique that combines low frequency ultrasound with a small electric current (electrosonophoresis). Portable instrumentation, positioned at the skin surface, delivers a brief burst of ultrasound and low electric field, creating a brief transdermal flux from underlying blood vessels into extracellular fluid and to the skin. The technique allows many analytes present in blood to be collected and measured at the skin surface. In humans, the method is without sensation and is innocuous. Actual blood removal is not required, and repeated measure over a short time period does not cause discomfort. In animals accustomed to human handling, no behavioural disturbance has been seen when using the instrumentation. Large cellular substances, such as red blood cells, cannot be collected by this method, but analytes, including hormones, sugars, free fatty acids, immunoglobulins and lactate, have been collected and measured with a high correlation ($r^2 \geq 0.89$) to blood levels over a wide physiological range of change. The low invasiveness of use, the removal of the need to extract actual blood fluid, and the low stressfulness of the technique, suggest that this may be a powerful tool for welfare studies.

Key words: *hormones, low invasive measurement, sonophoresis, transdermal exudate.*

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Introduction

An important experimental design constraint in use of animals is to minimise invasive load and accompanying stress. This is particularly so in studies aimed to improve the welfare of animals. A common confounding problem is that the experimental techniques may themselves elicit stress responses in the animal. In particular, many changes of interest are reflected in analytes in the blood. Collection of blood is often stressful and mildly noxious, and long-term catheterisation imposes an invasive load on the animal. Many stress-related changes are transient and easily missed or diluted down by infrequent sampling, yet more-frequent sampling and blood removal is also demanding on the animal.

Sampling blood-related analytes painlessly has received considerable research effort (1, 2), reflecting the potential usefulness of non-invasively obtained measures for clinical and laboratory use. Transdermal exudate, sampling analytes that have “migrated” from blood vessels to skin surface, has shown promise in the collection of a number of analytes particularly if enhanced by ultrasound (1–3). Blood glucose levels have been successfully tracked by using this sonophoresis technique (1). The method appears to work best with hydrophilic molecules, but combining an electric field with ultrasound (electrosonophoresis) may further improve the movement of analytes from blood to skin surface, particularly

those that are hydrophobic or complex in carrier charge (4). For animal and human experimentation, mindful of welfare and experimental ease, a painless and low-invasive method of sampling combined with the ability to measure changes relative to blood-borne levels would be an excellent addition to currently available experimental tools. Not only would it facilitate low invasive approaches, but also allow more-intensive data sampling, which conceivably could reduce animal numbers in an experiment.

The sheep and human studies reported here used non-invasive sampling of transdermal exudate facilitated by electrosonophoresis. Testosterone, cortisol, oestradiol and insulin were chosen as analytes because of their broad endocrinological roles and interest. The hormones were followed across an exercise stress, as this changes their levels in circulation in a short period. Glucose was chosen as a hydrophilic marker, again of broad relevance. A further study involving measurement in real-time of one of the analytes was also made.

Materials and Methods

Collection apparatus

A collection head of acetyl polyformaldehyde plastic was manufactured to fit tightly over an adapted

commercial hand-held ultrasound device (ITO). At the solid end of this head, a small chamber (300 μ l volume) was constructed, which had an entry and exit port feed by polyethylene tubing, allowing for a constant perfusion flow. An adapted semi-permeable dialysis membrane fitted over the chamber and was sealed with a rubber O-ring, making the chamber watertight. This end of the head made contact with the skin during sampling. On either side of the head, electrodes provided a 9V driven electric field. The other end of the sample head fitted tightly onto the modified ultrasound device (Figure 1).

Operation of the transdermal collection

Parameters for optimal transdermal collection were set following a series of pilot studies in sheep. A small amount of sodium lauryl sulphate/ethanol gel (SLS-EG) was rubbed into the skin area of collection 10 minutes prior to the procedure, and an initial application of ultrasound for 1 minute was given to start the transdermal flux. The chamber in the collecting head was filled with 10% ethanol; the head was then positioned against the skin surface of the subject, and 1 minute of ultrasound was applied (output 20kHz, 10W/cm² calculated at skin surface, pulsed 5 seconds on 5 seconds off, using an ITO physiotherapy and rehabilitation unit, Tokyo 176-8605, Japan). At the completion of 1 minute, fluid was allowed to flow through the chamber at a rate of 300 μ l/minute for a further minute while the device remained in contact with the skin. During the full 2 minutes contact with the skin, a 9V elec-

tric field was passed across the head, at the skin surface.

Sheep

Ten animals were penned and the jugular vein of each animal was catheterised. Animals were left to rest for 50 minutes, and then a single ultrasound application was applied for 1 minute to a shaved site on the back of each animal. The animals remained in the pens for 1 hour, before being run around a paddock by a human shepherd for 30 minutes and re-penned for a further hour. The animals were briefly restrained at regular intervals during this period, and a series of electrosonophoresis collections were made concurrently with blood collection.

Human

Ten human male volunteers were tested twice a week over a 3-week period. The experiment was conducted at a room temperature of 20–22°C. An initial 1-minute burst of ultrasound was made on the volar surface of the forearm, following application of the SLS-EG mix. Ten minutes later, and at regular intervals for the next 7 hours, transdermal (electro-sonophoresis) collection was made from this site on this forearm (see Figure 2), simultaneous to a venepuncture blood collection being made from the other. Subjects rested for 3 hours before exercising at moderate intensities, for 40 minutes (heart rates maintained in the range of 130–160bpm) on a rowing machine. The volunteers then warmed down lightly with stretching

Figure 1: The adapted ultrasound delivery device, the collecting chamber, membrane and O-ring



Figure 2: The transdermal collection device in use on a human arm



exercises for a further 10 minutes and rested for a further 3 hours. In a separate series of experiments on these human subjects, the transdermal and blood samplings were repeated as for the non-exercising period; however, on each transdermal sample, a different site on the volar forearm was used, ranging from wrist to elbow level. This was then repeated with the room temperature increased to 28–30°C.

Skin studies

In a separate study, four animals had skin biopsies removed under local anaesthetic to assess whether repeated exposure to the sampling technique led to histological changes in skin structure. Biopsies were taken after 10 days of sampling with 40 samples made on each day.

Real-time measurement

In other studies (5, 6), we have developed immunosensors that provide rapid measurement of very low concentrations of neurohumoral factors. In several studies, we combined measurements made on-line by such a sensor for testosterone with measurements made in the transdermal exudates off-line. A rapid on-line measurement would have considerable experimental durability.

Results and Discussion

Transdermally-collected exudate appeared to mirror blood changes with high consistency. Collection was made from subjects with relative ease and in human subjects without any report of pain or discomfort. Animals showed no obvious behavioural aversion to the procedure and had been familiarised to the overall experiment procedures. In repeated measures, without imposed stress, the transdermal method did not elevate cortisol, suggesting that it was low stress in nature.

As previously noted by others (1, 3, 4), a short initial application of ultrasound was needed to start transdermal flux. Once this flux reached a point of equilibration with blood levels, measurement closely reflected changes in blood levels. However, this close relationship was only maintained if a further application of ultrasound and electric field was applied at least every 30 minutes or as part of each measurement procedure. Unlike previous work, where at least 4 hours of transdermal glucose sampling was permitted following a single ultrasound application (1), hormone sampling appeared to require much more frequent reapplication of the ultrasound and electric field. This may reflect transdermal flux differences between hydrophilic

molecules, such as glucose and more complex structured molecules, such as hormones.

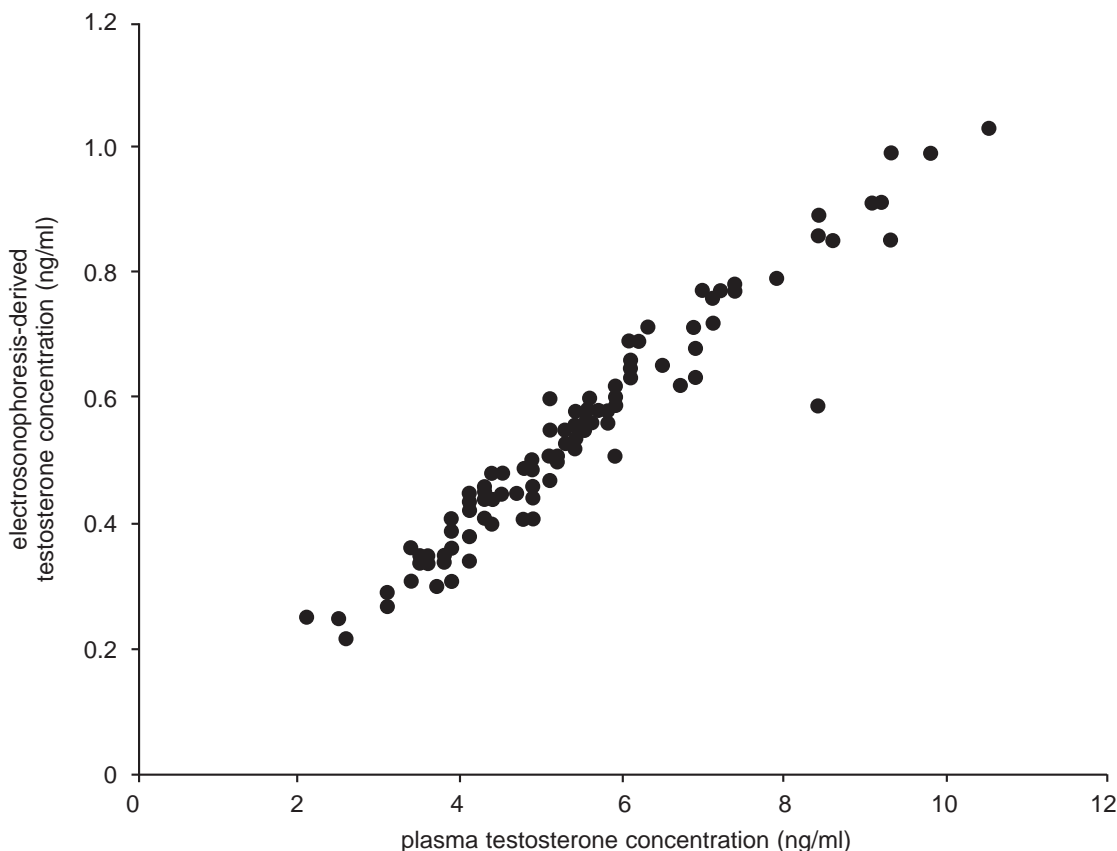
In the present studies, a 1-minute application of the ultrasound, when combined with the electric field, was sufficient to start transdermal flux, compared to a two-minute application in previous studies (1) with similar ultrasound power. Relative recovery in transdermal samples with ultrasound alone, compared to serum levels, was of the order of 4%, which was increased slightly with application of an electric field and substantially improved by the use of the collecting head device (recoveries between 9 and 15% in sheep; 8 and 12% in humans). In these studies, the correlations achieved for glucose, testosterone and cortisol were very high ($r^2 \geq 0.79$ in sheep; $r^2 \geq 0.82$ in humans) and provided valid prediction of blood levels (Figure 3).

Transdermal collection had a very low intra-subject variability in both sheep and humans. The inter-variation was much greater in the sheep than in humans. In the latter, this low inter-variation allowed good predictive measurement not only within but also across subjects. Position of the device, in humans, showed little contribution to variability, with most places on the volar forearm giving similar results. A high degree of intra-subject consistency but considerable inter-subject variability has been reported for ultrasound collection of glucose (1). In this previous study, variation in sampling site also contributed to variability within a subject (1). These were not such issues for human subjects in this present study, perhaps indicating a more robust sampling system. Room temperature was also controlled for all human subjects in our experiments, and this could conceivably influence sampling properties. An 8°C increase in room temperature was associated with a 1–3% trend toward higher recoveries.

Transdermal recovery was slightly less from sheep than humans, and with much greater inter-subject variability. This may reflect the different sampling sites in the two studies. In humans, the volar surface of the forearm offered a highly vascular area, while the ventral surface of the sheep's back did not, and skin layers differ in thickness. Preliminary data, not shown, suggest that heating the skin in sheep allows an improved recovery and reduced inter-subject variability. Other more vascular sites, such as the ear, also appear to further improve this in sheep. Inter-subject variability did not influence the usefulness of the device in this study, but it needs consideration in studies where absolute across-subjects calibration is needed.

The hormone levels, other than cortisol and insulin, in the sheep were low but typical of ewes outside of the breeding season (7, 8). Human subject levels were consistent with that expected for young fit men (9–12).

Both studies demonstrated changing concentrations of analytes in response to exercise. In sheep,

Figure 3: Correlating plasma and exudate (transdermal fluid) measures of testosterone

cortisol, testosterone (Figure 4) and glucose rose significantly ($p \leq 0.05$) with exercise, while insulin decreased ($p \leq 0.05$) and 17- β oestradiol showed little change. In humans, cortisol increased ($p \leq 0.05$) with exercise, but insulin decreased ($p \leq 0.05$). Testosterone and 17- β oestradiol were more variable, showing individual subject-related increases or decreases across exercise and recovery. Glucose increased ($p \leq 0.05$) across exercise and recovery.

Sonophoresis, the use of ultrasound, as a transdermal facilitator of certain analytes, such as glucose, has been demonstrated as a useful concept for sampling previously (1–3). In the studies presented, the addition of an electric field (electrosonophoresis), and a specialised collection head, enabled a good collection of hormones, in terms of relativity to blood levels, using similar short bursts of ultrasound. Hormones are complex molecules, often with accompanying charge and water-insoluble. The addition of an electric field appeared facilitative in their movement, while the arrangement of the collecting head seemed to improve further the collection for measurement.

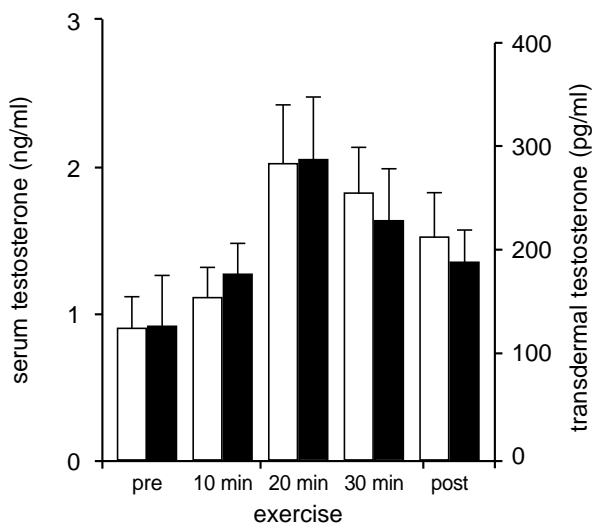
In studies employing the immunosensor, on-line readings of testosterone levels obtained in real-time

correlated ($r^2 < 0.99$) with those measured off-line (Figure 5). For this hormone, real-time measurement thus seems a possibility. This may not be the case for all analytes and will depend on the relative amount recovered. However, real-time measures offer a further experimental sophistication to the use of sonophoresis as a technique.

Conclusions

The electrosonophoresis technique appears very useful as a non-invasive sampling tool. It is portable, handy and easy to use in animal studies. No evidence of skin damage, either *in vivo* or in histological examination of biopsies made from the sampled area, was seen. In addition, there were no signs or reports of either pain or aversion with repeated measurement. Preliminary work, including data not yet presented, suggests that this method is applicable to a broad range of animal species, including rats, cattle, cats, dogs, horses, birds and sharks. There are numerous benefits that such a method would also offer human and other animal endocrine studies, including addressing welfare and stress concerns surrounding invasive sampling.

Figure 4: Changes in concentration of testosterone measured in plasma and exudate from sheep before, during and after exercise



□ = serum testosterone; ■ = transdermal testosterone.

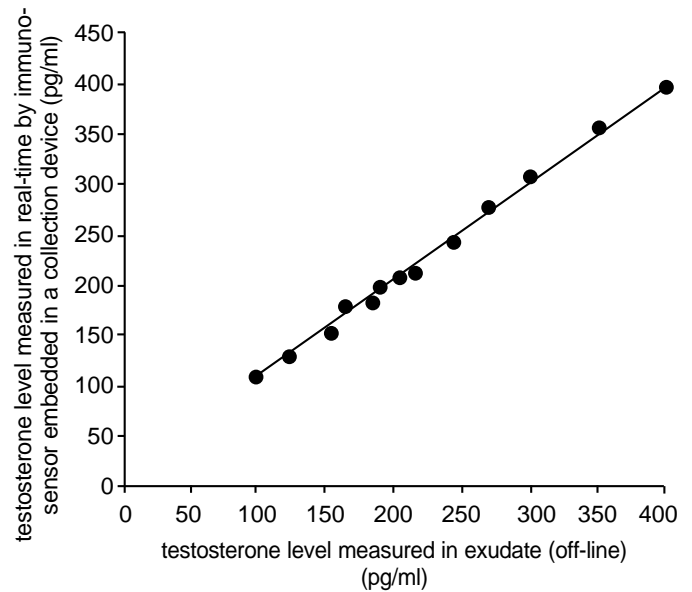
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Figure 5: Correlating real-time and off-line exudate measures of testosterone



$r^2 = 0.99$.

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