



Goodhead, L., & MacMillan, F. (2017). Measuring osmosis and hemolysis of red blood cells. *Advances in Physiology Education*, 41(2), 298-305. <https://doi.org/10.1152/advan.00083.2016>

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1 MEASURING OSMOSIS AND HAEMOLYSIS OF RED BLOOD CELLS

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12

13 **ABBREVIATED TITLE**

14 Measuring osmosis and haemolysis of red blood cells

15

16 **KEYWORDS**

17 Haematocrit

18 Handing tissue fluids

19 Osmolarity

20 Tonicity

21

22 **ABSTRACT**

23 Since the discovery of the composition and structure of the mammalian cell membrane, biologists have had
24 a clearer understanding of how substances enter and exit the cell's interior. The selectively-permeable nature
25 of the cell membrane allows the movement of some solutes and prevents the movement of others. This has
26 important consequences for cell volume and the integrity of the cell and, as a result, is of utmost clinical
27 importance, for example in the administration of isotonic intravenous infusions. The concepts of osmolarity

28 and tonicity are often confused by students as impermeant isosmotic solutes such as NaCl are also isotonic;
29 however, isosmotic solutes such as urea are actually hypotonic due to the permeant nature of the
30 membrane. By placing red blood cells in solutions of differing osmolarities and tonicities, this experiment
31 demonstrates the effects of osmosis and the resultant changes in cell volume. Using haemoglobin standard
32 solutions, where known concentrations of haemoglobin are produced, the proportion of haemolysis and the
33 effect of this on resultant haematocrit can be estimated. No change in cell volume occurs in isotonic NaCl,
34 and by placing blood cells in hypotonic NaCl incomplete haemolysis occurs. By changing the bathing solution
35 to either distilled water or isosmotic urea, complete haemolysis occurs due to their hypotonic effects. With
36 the use of animal blood in this practical, students gain useful experience in handling tissue fluids and
37 calculating dilutions and can appreciate the science behind clinical scenarios.

38

39 **INTRODUCTION**

40 **Objectives and Overview**

41 The movement of water and small molecules across the selectively-permeable membranes of mammalian
42 cells is a fundamental concept of physiology. These processes can be difficult for students to visualise and
43 appreciate and it is often left to images in textbooks or online animations to explain such movements. This
44 practical uses animal blood bathed in solutions with differing osmolarities and tonicities to explore the
45 concept of water movement by osmosis and the resultant haemolysis that can occur when red blood cells
46 are exposed to hypotonic solutions. Students are given the opportunity to handle body fluids, practise
47 preparing dilutions and make accurate observations.

48

49 **Background**

50 In 1925, Gorter and Grendel (6) were the first to report the bilayer nature of the cell membrane. The structure
51 of the cell membrane was further advanced by the work of Singer and Nicolson (18) who described the
52 presence and location of proteins in the bilayer and developed the fluid mosaic model. In the mammalian
53 cell membrane, the phospholipid bilayer alone is permeable to some substances such as oxygen, a small non-
54 polar molecule, and partially permeable to water but some substances such as charged ions and glucose are

55 impermeant without the additional presence of protein channels and transporters in the membrane. The
56 combined properties of the phospholipid and proteins has resulted in the use of the term the 'selectively-
57 permeable' membrane (3,9). The extent to which solutes can cross the cell membrane dictates the tonicity
58 of extracellular fluids and therefore the size and shape of cells from the resultant osmotic water movement
59 (19). Knowledge of the structure and function of cell membranes and the movement of substances across
60 the membrane is fundamental to all biomedical science disciplines and is often taught in early parts of
61 undergraduate courses.

62

63 Osmosis is the movement of water down its osmotic gradient across a selectively-permeable membrane (5).
64 The establishment of an osmotic pressure gradient, i.e. the pressure required to prevent the movement of
65 water down its gradient, is a result of the difference in numbers of impermeant particles in solution on either
66 side of the membrane (14). Water can move directly through the cell membrane; however, due to the lipid
67 bilayer nature of the membrane this process is relatively slow. It was the discovery of water carrying pore-
68 forming proteins known as aquaporins (16) that helped improve knowledge of how water moves from
69 intracellular to extracellular fluid and vice versa. Water balance is crucial in homeostasis; hormones such as
70 antidiuretic hormone (ADH) and atrial natriuretic peptide (ANP) are released in response to changes in
71 plasma composition and volume respectively, and act on the kidney to regulate plasma osmolarity and
72 volume.

73

74 The osmolarity of a solution is determined by the total number of particles present, known as osmolyte
75 particles, and is not affected by the identity of these molecules (19). The higher the osmolarity of a solution,
76 the greater the concentration of osmolytes and the physical properties of a solution such as osmotic pressure
77 and freezing point will be dependent on the concentration of osmolytes in solution. Osmolarity is calculated
78 from the sum of the molar concentration of each solute multiplied by the osmotic coefficient for that solute.
79 The osmotic coefficient is determined by the degree to which a solute (e.g. an ionic compound) dissociates
80 in solution therefore an osmotic coefficient of '1' indicates that the solute completely dissociates in solution.

81 For example, to calculate the osmolarity of a 0.9% weight/volume NaCl (MW 58.44) solution firstly the
82 molarity is calculated by:

83

84 Molarity of a % w/v solution (M) = % solution in g/litre ÷ molecular mass of the solute

$$85 \quad 0.154 \text{ M} = 9 \text{ g/litre} \div 58.44 \text{ g/mol}$$

86

87 To calculate the osmolarity, given that NaCl dissociates into two ions (Na⁺ and Cl⁻) in solution and has an
88 osmotic coefficient of 0.93, the following equation is used:

89 Osmolarity of solution (osmol/l) = molarity (M) x number of osmoles produced by dissociation x osmotic
90 coefficient

$$91 \quad 0.286 \text{ osmol/l or } 286 \text{ mosmol/l} = 0.154 \text{ M} \times 2 \times 0.93$$

92

93 Osmolarity and tonicity are often used interchangeably by students but they are not the same. Tonicity refers
94 to the effect a solution has on cell volume as a result of the permeability of the membrane to that solute.
95 Tonicity is therefore determined by the osmolarity and whether the solute can cross the cell membrane; it is
96 the concentration of the impermeant solutes alone that determines tonicity. When comparing fluid
97 concentrations to that of extracellular body fluid, the terms isotonic, hypertonic and hypotonic are used
98 rather than osmolarity as they describe the effect the solution has on cell volume which is of physiological
99 significance. The tonicity will result in: no net movement of water (isotonic), net flow of water out of a cell
100 (hypertonic), or net flow of water into a cell (hypotonic). Two solutions that are isosmotic may not be isotonic.
101 A key example is isosmotic urea and isosmotic NaCl. Both urea and NaCl have the same osmolarity, having
102 the same total number of osmolyte particles, however the membrane is permeable to urea which will freely
103 diffuse across the cell membrane and impermeable to NaCl. An isosmotic urea is therefore hypotonic
104 compared to an isosmotic and isotonic solution of the impermeant NaCl. As a result, the volume of a cell is
105 determined by the solution in which it is being bathed **and** whether the cell's membrane is permeable to the
106 solute. If a membrane is not equally permeable to all solutes then a difference in water movement will be
107 observed that is not explained by osmolarity alone and hence an additional term, tonicity, is required.

108 Hypotonic solutions lead to cell swelling and eventual rupture or lysis if the resultant osmotic movement of
109 water is great enough. In the case of red blood cells this is referred to as haemolysis (4).

110

111 Knowledge of osmosis and tonicity is crucial in understanding the movement of fluids in the body. These
112 concepts are fundamental in normal physiological processes; one example is that of water reabsorption in
113 the kidney as increases in osmolarity are detected by the hypothalamus and stimulate the secretion of ADH
114 resulting in greater water retention and excretion of more concentrated urine (7). Osmosis and tonicity are
115 important clinically as the failure of the body to respond to changes in osmolarity, or the failure to release
116 ADH, result in the condition diabetes insipidus. Another important concept is the diagnosis of the different
117 types of dehydration and the administration of appropriate intravenous fluids (2). In this practical, using easy
118 to obtain red blood cells as model cells (1), students can explore the concepts of membrane permeability,
119 osmosis, osmotic pressure, tonicity and haemolysis whilst also learning key laboratory skills such as making
120 dilution series and handling tissue fluids.

121

122 **Learning Objectives**

123 After completing this activity, the student will be able to:

- 124 1. CONTENT KNOWLEDGE: Define key terms used in explaining concentration, osmolarity, osmotic
125 pressure and tonicity
- 126 2. CONTENT KNOWLEDGE: Calculate the osmolarity of a solution
- 127 3. CONTENT KNOWLEDGE: Describe and explain the consequences of bathing red blood cells in
128 solutions of differing tonicity
- 129 4. PROCESS SKILLS: Handle mammalian blood samples safely
- 130 5. PROCESS SKILLS: Prepare standard saline solutions
- 131 6. PROCESS SKILLS: Measure haematocrit and estimate haemoglobin concentration
- 132 7. PROCESS SKILLS: Carry out experiments with careful planning, accurate observation and recording of
133 results

134

135 **Activity Level**

136 This activity is used to teach students in their first year of undergraduate study in physiology. This practical
137 is used on our Physiological Sciences programme and Veterinary Science programme but would also be
138 suitable for other biomedical science or healthcare professional programmes such as medicine.

139

140

141 **Prerequisite Student Knowledge or Skills**

142 Before undertaking this activity, students should have a basic understanding of:

143 Homeostasis and the proportions of fluid in intracellular and extracellular compartments

144 The definition of a solute, a solvent and a solution

145 The concept of osmosis and the movement of water across a selectively-permeable membrane

146

147 Students should know how to:

148 Perform basic calculations to work out volumes required for concentrations

149 Use pipettes to create serial dilutions from stock solutions

150 Collect data carefully and accurately

151 Observe safe laboratory practices

152

153 **Time Required**

154 This practical is run in a 3 hour laboratory time-slot. The practical is completed within one session; however,
155 it is expected that students complete their pre-reading of the laboratory notes which explain the concepts of
156 osmolarity, tonicity and how to calculate osmolarity (to aid in achieving content learning objectives 1 and 2)
157 and an online pre-practical quiz before they come to the practical. This preparation work is expected to take
158 around 1 hour.

159

160 **METHOD**

161 **Equipment and Supplies**

162 The following equipment and supplies are needed:

163 Solutions

164 1. Distilled water (20 ml per pair of students)

165 2. 2.7% w/v NaCl solution (2.7 g NaCl per 100 ml of distilled water) (20 ml per pair of students plus that
166 required for non-haemolysed blood preparation). This stock solution is used to prepare all other NaCl
167 solutions in the experiment

168 3. Isosmotic urea solution (17.1 g/l) (5 ml per pair of students plus that required for haemolysed blood
169 preparation)

170 4. Fresh mammalian blood. This blood is referred to for the rest of the experiment as non-haemolysed
171 blood. We find that there are no appreciable differences in the outcome of the experiment depending
172 on which species blood is used although values of haemolysis can vary. Obtaining mammalian blood
173 supplies can be problematic if obtained locally direct from an abattoir; however, blood can also be
174 purchased online for example <http://www.rockland-inc.com/blood-products.aspx>. For a class of around
175 200 students working in pairs, approximately 1.5 L of blood is required (approximately 11 ml blood per
176 pair of students and allowing extra for repeat experiments if required). The blood must be heparinised
177 before use to prevent clotting by the addition of heparin sodium (5,000 I.U/ml per 1.5 litres blood). This
178 blood is then used to produce the haemolysed and non-haemolysed blood as follows:

179 5. Haemolysed blood. To prepare the haemolysed blood in manageable volumes, 250ml of non-
180 haemolysed blood is measured into a 600ml beaker together with 250 ml urea solution (17.1g/l) and
181 stirred. The tonicity of the urea and resultant osmotic water movement results in haemolysis of the cells
182 and this will form the blood used for the production of the haemoglobin standards that will be used to
183 assess the degree of haemolysis in the experiment. Decant 10 ml of the haemolysed blood into 50
184 centrifuge tubes (one per pair of students), labelled H for haemolysed blood and centrifuge at 6000 rpm
185 for 2 minutes. Repeat depending on quantities of blood required i.e. if 1l required repeat once.

186 6. Non-haemolysed blood. To prepare the non-haemolysed blood in manageable volumes, 275 ml of non-
187 haemolysed blood (from the original heparinised fresh mammalian blood) is prepared by the addition
188 of 275 ml of 0.9% w/v saline and stirred gently. This forms the non-haemolysed blood which will be used

189 for the main part of the experiment at an equal concentration to the haemolysed blood. Decant 11 ml
190 of the non-haemolysed blood into 50 centrifuge tubes (one per pair of students) labelled N for non-
191 haemolysed blood. Repeat depending on quantities of blood required i.e. if 1l required repeat once.

192 An assumption is made that the haemoglobin concentration of the original blood sample is 15 g/dl; but
193 as the haemolysed blood is diluted 1:1 with isosmotic urea (17.1g/l) and the equivalent non-haemolysed
194 blood is diluted 1:1 with isosmotic (0.9% w/v NaCl), the haemoglobin concentration of both blood
195 samples is therefore assumed to be 7.5 g/dl (75 g/litre).

196 Equipment

- 197 1. 600 ml glass beakers (2 for blood preparation)
- 198 2. 500 ml measuring cylinders (2 for blood preparation)
- 199 3. Stirring rods (2 for blood preparation)
- 200 4. 25 ml glass beakers for water, 2.7% w/v NaCl and urea distribution (3 per pair of students)
- 201 5. 1.5 ml plastic Eppendorf tubes with hinged cap (11 per pair of students)
- 202 6. 10 ml plastic centrifuge tubes with cap (10 per pair of students)
- 203 7. Centrifuge tube racks (1 per pair of students)
- 204 8. 75 µl glass microhaematocrit tubes (Hawksley catalogue no. 01603) (6 per pair of students)
- 205 9. Plasticine
- 206 10. Centrifuge with centrifuge tube rotor and microhaematocrit tube rotor (Hettich EBA21 centrifuge with
207 1416 rotor and 1450 haematocrit rotor)
- 208 11. Haematocrit readers (Hawksley) or 30 cm rulers (a number of readers/rulers can be shared between
209 pairs of students)
- 210 12. 1.5 ml disposable plastic pipettes or equivalent Gilson pipettes if available (3 disposable pipettes per
211 pair of students)
- 212 13. Marker pens (1 per pair of students)
- 213 14. White paper (1 sheet per pair of students)

214

215 **Human or Animal Subjects**

216 The animal blood used in this experiment is obtained as a by-product from a local abattoir and therefore the
217 animals are not slaughtered for the purpose of this experiment.

218

219 **Instructions**

220 *Preparation prior to the practical*

221 In advance of the class, students must calculate (1) the volume of distilled water and 2.7% w/v NaCl stock
222 solution required to produce 9 ml each of 0.9 and 0.45% w/v saline solutions; (2) the volumes of haemolysed
223 blood and 0.9% w/v NaCl (ml) required to produce 1.5 ml % haemoglobin concentrations and (3) the
224 haemoglobin concentration (g/dl) in results tables 1-3 provided in their lab books.

225

226 In our programmes this is the first practical that students will have had to calculate and make serial dilutions
227 and handle blood; two key, but challenging, transferable skills.

228

229 *Making saline solutions and haemoglobin standards*

230 We recommend that students carry out this practical working in groups of two or three. Students begin the
231 practical by making a set of standard solutions of haemolysed blood of known haemoglobin concentration
232 to use later in the experiment which they will compare against the unknown haemoglobin containing
233 solutions they will produce. Haemolysed blood is used to create these haemoglobin standards as this contains
234 red blood cells that have already fully lysed in urea and all the haemoglobin has been released into the
235 solution. The steps below take the students through the practical:

236 1. Using the 2.7% w/v NaCl and 1.5 ml pipettes provided, prepare 9 ml each of 0.9% w/v NaCl and 0.45% w/v
237 NaCl solutions in two labelled 10ml plastic centrifuge tubes from the dilutions calculated below in Table 1
238 (values underlined are calculated by the students in advance of the class).

239

240 **Table 1: Dilutions calculations for saline solutions**

241

242 2. Using the marker pen, label five Eppendorf tubes – 100%, 66%, 33%, 7% and 1% which will represent the
243 percentage of haemolysed blood to be added to these Eppendorf tubes. Using the volumes calculated in
244 Table 2, use 1.5 ml pipettes to add the appropriate volumes of 0.9% w/v NaCl solution and haemolysed blood
245 to each labelled Eppendorf tube (values underlined are calculated by the students in advance of the class).
246 Gently invert the tube containing the haemolysed blood before use to ensure the blood is evenly mixed and
247 once filled also invert each Eppendorf tube to ensure mixing.

248

249 **Table 2: Dilutions calculations for haemoglobin standards from haemolysed blood**

250

251 The calculations performed in Table 3 provide reference haemoglobin concentrations for each haemoglobin
252 standard (values underlined are calculated by the students in advance of the class).

253

254 **Table 3: Haemoglobin concentration in each standard solution**

255

256 3. Lay out the five mixed haemolysed blood/0.9% w/v NaCl solutions in the Eppendorf tubes on a blank sheet
257 of white paper to observe the colours. The colours of the haemoglobin standards should range from
258 translucent pink to translucent red and should look similar to Figure 1. These haemoglobin standards will be
259 used later in the experiment and should be kept to one side until then.

260

261 **Figure 1: The haemoglobin standards produced from haemolysed blood with 1% haemolysed blood to the**
262 **left of the image and 100% haemolysed blood on the right. Image courtesy of the University of Bristol.**

263

264 *Investigating the effects of tonicity on red blood cells*

265 The next part of the experiment investigates the effects of membrane-permeable and membrane-
266 impermeable solutions of differing concentrations on whole red blood cells using the non-haemolysed blood
267 sample. Both haematocrit and % haemolysis will be estimated. The haematocrit will indicate the degree to
268 which red blood cells swell or shrink when exposed to the different solutions but does not take into account

269 if haemolysis has occurred. Percentage haemolysis gives a measure of the degree of haemolysis of the
270 samples and can be used to determine if red blood cells have swollen and burst. Haematocrit alone cannot
271 distinguish between cell shrinkage and a combination of swelling and lysis.

272 4. Label an additional six 10 ml plastic centrifuge tubes from 1-6. Gently invert the tube containing the non-
273 haemolysed blood several times before use to ensure an even suspension of red blood cells.

274 5. Prepare the centrifuge tubes as follows:

275 Tube 1 – 1.5 ml non-haemolysed blood + 1.5 ml 2.7% w/v NaCl

276 Tube 2 – 1.5 ml non-haemolysed blood + 1.5 ml 0.9% w/v NaCl

277 Tube 3 – 1.5 ml non-haemolysed blood + 1.5 ml 0.45% w/v NaCl

278 Tube 4 – 1.5 ml non-haemolysed blood + 1.5 ml distilled water

279 Tube 5 – 1.5 ml non-haemolysed blood + 1.5 ml isosmotic urea

280 Tube 6 – 3 ml non-haemolysed blood

281 When filled, gently invert the centrifuge tubes several times to ensure the blood is mixed and leave for 10
282 minutes before proceeding with the next step.

283 6. The blood solutions are then prepared for centrifuging to allow the measurement of the packed cell volume
284 (haematocrit) of each sample. Label six glass microhaematocrit tubes 1-6 to correspond to the samples in the
285 plastic centrifuge tubes. In turn, invert each centrifuge tube several times to ensure even dispersal of red
286 blood cells and then dip the corresponding microhaematocrit tube in the blood until capillary action has filled
287 the glass tube. Seal the bottom of the microhaematocrit tube with a small plug of plasticine by twisting the
288 bottom of the tube in a tray of plasticine.

289 7. Centrifuge the microhaematocrit tubes at 6,000 rpm for 2 minutes using the microhaematocrit tube rotor
290 until the cells have packed together at the bottom of the tube leaving the fluid (supernatant) above.

291 It is not expected that the centrifuges are operated by the students, in our laboratory students bring their
292 samples to the shared laboratory centrifuges and these are run by experienced demonstrators or technicians.

293

294 *Measuring haematocrit*

295 8. After centrifuging, measure the haematocrit of each sample using a haematocrit reader and read off the
296 % haematocrit. If haematocrit readers are difficult to obtain, a ruler can be used instead. By this method,
297 measure the total length of the column of fluid and the length of the column of packed cells and calculate
298 the proportion of the total column that is made up of packed cells at the bottom. This percentage is the
299 haematocrit. Record the haematocrit readings in the *observed haematocrit* column in Table 4.

300

301 **Table 4: Haematocrit measurements for non-haemolysed blood**

302

303 9. With the exception of tube 6, the haematocrit readings measured are for blood diluted 50:50 with a saline
304 solution. Therefore complete the *dilution factor* column with a dilution factor of 2 for tubes 1-5 and a dilution
305 factor of 1 for tube 6. To calculate the true haematocrit values, complete the final *corrected haematocrit for*
306 *non-haemolysed blood* column in Table 4 by using the following equation:

307

$$308 \quad \text{Corrected haematocrit} = \text{observed haematocrit} \times \text{dilution factor}$$

309

310 *Estimating haemolysis*

311 10. Following the measurement of haematocrit, estimate the percentage of haemolysis of the red blood cells
312 in the various solutions. To do this, centrifuge the remaining contents of the 6 plastic centrifuge tubes at
313 6000 rpm for 2 minutes using the centrifuge tube rotor. Take six clean 1.5 ml plastic Eppendorf tubes also
314 labelled 1-6, pipette 1.5 ml of the supernatant from each correspondingly labelled centrifuge tube into the
315 labelled Eppendorf tube taking care not to disturb the red blood cell pellet at the bottom of the tube.

316 11. The colours of the six samples of supernatant can then be compared to that of the known haemoglobin
317 standard solutions prepared at the beginning of the practical. Using the colours of the known haemoglobin
318 standard solutions as a scale, estimate the concentration of observed supernatant haemoglobin with the
319 darker the colour of the sample indicating the greater the amount liberated haemoglobin in the supernatant
320 and hence the greater degree of haemolysis. Using the known haemoglobin concentrations (g/dl) calculated

321 in Table 3, record these observations for tubes 1-6 in the *observed supernatant [Hb]* column of Table 5. If the
322 colours are not exact matches, estimate whereabouts between the two standards the concentration falls.
323 12. To convert the observed haemoglobin concentration into an estimated percentage of haemolysis of the
324 red blood cells, with the exception of the non-haemolysed blood sample which contained 7.5 g/dl
325 haemoglobin, the blood in the other mixtures was diluted 50:50 and therefore contained half the original
326 haemoglobin. To estimate the amount of haemolysis that occurred in each sample use the following
327 calculations and complete the *estimated % haemolysis* column of Table 5:

328

329 **Diluted samples estimated % haemolysis = [observed supernatant Hb concentration / 3.75] x 100**

330 **Non-haemolysed blood estimated % haemolysis = [observed supernatant Hb concentration / 7.5] x 100**

331

332 13. The corrected haematocrit recorded in Table 4 was generated by non-haemolysed red blood cells only as
333 these were the whole cells that would have made up the packed cell volume in the haematocrit tubes. To
334 correct for haemolysis in each sample and allow an estimate of what haematocrit would be had there had
335 been no cell lysis, use the following calculation and complete the final column (*corrected haematocrit*) of
336 Table 5:

337

338 **Corrected haematocrit (%) = [100 / (100 – estimated % haemolysis)] x corrected haematocrit for non-**
339 **haemolysed blood (%)**

340

341 **Table 5: Estimated haemolysis and final corrected haematocrit**

342 14. When all data have been collected, each group should pool their final corrected haematocrit (%) data
343 from Table 5 with the rest of the class using a spreadsheet on a central computer to ensure that group data
344 can be distributed for more comprehensive analysis following the class.

345

346

347 **Troubleshooting**

348 A common student mistake in this practical is the incorrect or lack of labelling of tubes and pipettes
349 containing the different solutions during the various steps undertaken. As a result, students lose track of the
350 contents of tubes they are testing and find their results are meaningless. This is an important error to impress
351 upon the students as, if this is kind of mistake occurs in a clinical setting, the outcome could be life-
352 threatening. Trained demonstrators should be on hand to spot mistakes early and help students rectify them
353 as soon as possible.

354

355 Students also often find it difficult to perform the correct calculations to work out dilutions (15). It is
356 recommended that students are encouraged to attempt these calculations (Tables 1-3) prior to the practical
357 and come prepared to have these calculations checked by a demonstrator in the practical before proceeding.

358

359 **Safety Considerations**

360 Despite the risk to humans from animal blood being extremely low, when dealing with blood, standard safety
361 precautions must be taken to minimise the risk of infection. At all times in the laboratory general laboratory
362 safety rules must be followed including wearing a laboratory coat and using disposable gloves and hand
363 washing before leaving the laboratory. Any spilled blood or fluids must be wiped up immediately and
364 disposed of in waste bags provided. All sharps should be disposed of in a sharps box.

365

366 Unless the students are already trained and experienced in using centrifuges, the centrifuge should only be
367 operated by trained personnel and students should not be left to spin their samples unsupervised. The
368 centrifuge should be inspected for damage regularly. When using the centrifuge, ensure the tubes are
369 undamaged, firmly sealed and haven't been overfilled. When placing the tubes in the rotor they must be
370 balanced and the lid must never be opened while the rotor is moving. The centrifuge should not be left
371 unattended during use.

372

373

374 **RESULTS**

375 **Expected Results**

376 *Non-haemolysed blood*

377 Tube 6 which contains the non-haemolysed blood sample prepared in step 5 should be used as a control and
378 reference point against which to compare any changes to haematocrit in the other blood samples (tubes 1-
379 5) that were exposed to permeant and non-permeant solutes. Completed sample data tables (Tables 6 and
380 7) are given here from experiments carried out using pig blood but caution should be taken when making
381 direct comparisons to the values obtained as, although the relative changes should be the same, the actual
382 values can vary greatly depending on the blood sample used.

383

384 *The effects of hypertonic NaCl*

385 In step 5, non-haemolysed blood was exposed to 2.7% w/v NaCl solution which has an osmolarity of 859
386 mosmol/l and is hypertonic relative to plasma (tube 1). When red blood cells are placed in a hypertonic
387 solution, the higher effective osmotic pressure of the bathing solution compared to the intracellular fluid
388 results in water moving down its osmotic gradient and a net movement of water out of the cell via osmosis
389 (10). The red blood cells therefore lose their normal biconcave shape and shrink or crenate. This collapse
390 leads to a decrease in the packed cell volume, or haematocrit, of the solution in comparison to that of the
391 non-haemolysed blood as the cells take up less space due to the rapid loss of water. Very little haemolysis of
392 the red blood cells in the solution should be observed as no cells have taken on an additional water load and
393 burst or haemolysed; however, a few cells may have been damaged during handling and release some
394 haemoglobin.

395

396 *The effects of isotonic NaCl*

397 In step 5, non-haemolysed blood was exposed to an isotonic solution of 0.9% w/v NaCl (osmolarity 286
398 mosmol/l) (tube 2). This environment has an even distribution of osmolyte particles across both sides of the
399 cell membrane as intracellular fluid also has an osmolarity around 286 mosmol/l. There is therefore no net
400 water movement between the bathed red blood cells and the NaCl solution. The haematocrit of the solution

401 should be unaffected and the value similar to that of the non-haemolysed blood. Similarly, little if any
402 haemolysis of the red blood cells should have occurred.

403

404 *The effects of hypotonic NaCl*

405 In step 5, non-haemolysed blood was exposed to a low osmolarity (143 mosmol/l) hypotonic solution (0.45%
406 w/v NaCl) (tube 3). When red blood cells are exposed to these conditions where there is a higher
407 concentration of water and lower effective osmotic pressure outside the cell compared to the intracellular
408 fluid, this results in net movement of water into the cells via osmosis (11). The cells will increase in size and
409 some may haemolyse. In this sample therefore a small proportion of haemolysis should have been observed
410 with increased haemoglobin in the supernatant when compared to the whole blood and the remaining cells
411 which hadn't lysed would increase in size causing the haematocrit to increase.

412

413 *The effects of distilled water*

414 In step 5, the cells in tube 4 that were bathed in distilled water underwent complete haemolysis and the
415 estimated % haemolysis should have been 100%. With no ions present in the bathing solution this solution
416 was very hypotonic resulting in net movement of water into the red blood cells via osmosis causing all the
417 cells to lose the integrity of their membranes and to haemolyse releasing haemoglobin into the supernatant,
418 hence the strong red colour of the sample. The resultant corrected haematocrit was 0% as there were no
419 remaining complete red blood cells to contribute to pack cell volume. Comparing the results of distilled water
420 (tube 4) and 0.45% w/v (tube 3) is a clear example of how the osmotic fragility or susceptibility of red blood
421 cells to haemolysis depends on the degree of hypotonicity of the bathing solution.

422

423 *The effects of isosmotic urea*

424 In contrast to NaCl, the membrane is permeable to urea. In Step 5 when red blood cells were bathed in
425 isosmotic urea (286 mosmol/l) (tube 5), the effects of the permeability of the membrane to urea on both
426 haematocrit and degree of haemolysis were very different than when red blood cells are exposed to
427 isosmotic NaCl (tube 2). In the presence of an isosmotic urea solution, the red blood cells underwent

428 complete haemolysis with a corrected haematocrit of 0%. This is because although isosmotic, the urea
429 solution is not isotonic as urea can freely diffuse across the cell membrane into the cell via passive diffusion
430 and through urea transporters (20, 21). This leads to a change in cell volume as a result of osmotic water
431 movement (13). The isosmotic urea solution is therefore hypotonic because the reflection coefficient of the
432 membrane (permeability) for urea is 0.024 compared to a reflection coefficient of the membrane of 0.3 for
433 NaCl. If the membrane is completely impermeable to a solute the reflection coefficient would be 1. The
434 consequence of this is that the effective osmotic pressure of a urea solution is lower than that of NaCl of the
435 same osmolarity and, as a result, the osmotic gradient across the cell membrane is increased and water
436 moves into the red blood cells via osmosis causing the cell membrane to rupture and the cell to haemolyse.
437 Conversely, NaCl dissociates into Na^+ and Cl^- particles that cannot cross the cell membrane and therefore
438 generate an equal effective osmotic pressure between the extracellular fluid and the intracellular fluid. Under
439 these conditions the osmotic gradient across the cell membrane is maintained and the solution is both
440 isosmotic and isotonic. The same strong red colour of the urea sample in tube 5 should have been observed
441 as that of the distilled water sample in tube 4 as there is 100% haemolysis and 0% corrected haematocrit.

442

443 *Conclusions*

444 The observations and conclusions that should have been drawn from this practical are fundamental to
445 understanding basic cell physiology. A good grasp of the concepts covered by this practical will help students
446 appreciate the fact that cell membranes are indeed selectively-permeable and that the tonicity and
447 osmolarity of fluids affect cell size and structure. This is essential in understanding the concept of
448 homeostasis and will be referred to in later parts of many physiology courses including during study of the
449 gastrointestinal tract, regulation of NaCl by the nephron in the renal system and in particular the effect of
450 dehydration on the whole body.

451

452 Caution must be taken with the practical to ensure students observe the expected results, common mistakes
453 such as poor labelling of samples and contamination with urea due to pipette confusion can lead to students

454 obtaining results that may not be as anticipated. Careful supervision of students and pooling of data to
455 analyse the class averages should help prevent this.

456

457 **Table 6: Completed sample results of haematocrit measurements for non-haemolysed blood**

458

459 **Table 7: Completed sample results of estimated haemolysis and final corrected haematocrit**

460

461 **Figure 2: Mean corrected haematocrit values of blood following exposure to permeable and non-**
462 **permeable solutions (n=64). Error bars represent SEM.**

463

464 **Misconceptions**

465 From early on in many physiology-based courses, students struggle with the concepts of osmolarity and
466 tonicity and find it difficult to relate them to the direction of water movement. This practical can help
467 students to visualise different solutions and the effects that these can have on red blood cells. By being able
468 to see the colour change of the non-haemolysed blood samples mixed with various solutions they can relate
469 the theory of osmosis to what has happened to the cells when water moves out of the red blood cells in a
470 hypertonic solution (tube 1), when there is no net movement of water in an isotonic solution (tube 2) and
471 when water moves into the cells in a hypotonic solution (tube 3). The practical also helps students with
472 misconceptions surrounding haemolysis and haematocrit. The students often mistake the contents of the
473 tubes (4 and 5) containing distilled water and urea as having 100% haematocrit due to the dark colouration
474 of the whole sample with little or no visible plasma band rather than the product of 100% haemolysis due to
475 the effects of the tonicity. Trained demonstrators should be on hand to ensure that students are able to
476 relate their findings to the learning outcomes and a whole class tutorial on the outcomes of the experiments
477 and their meanings is scheduled for one week after the practical class.

478

479 **Evaluation of Student Work**

480 As discussed, there are a number of ways in which students may not get the results expected. To ensure that
481 students have access to some representative data on which to perform any post-practical analysis, students
482 are expected to pool their results with the rest of the group to produce group data for the class prior to the
483 finish and this is then shared online with the students to use immediately after the class.

484

485 We assess our students on this work through the submission of an online post-practical assessment. This
486 takes the form of multiple choice questions on the background physiology, method, results and physiological
487 significance of the findings. Questions included in the assessment test whether students have grasped the
488 direction of fluid movement in the presence of different solutions and the physiological reasons for this. With
489 regards to further data analysis, students are expected to plot the class findings in graphical form. They are
490 instructed to produce a column/bar graph of the final corrected haematocrits (%) from all solutions with the
491 error bars as the standard error of the mean and this is uploaded as part of their online post-practical
492 assessment (see Figure 2). Students are also given information on how to calculate osmolarity and are
493 expected to perform calculations themselves.

494

495 **Inquiry Applications**

496 As this practical is run at the start of the students' exploration of physiology at undergraduate level there is
497 very limited inquiry in this practical and would be considered 'Methods' level. The questions being explored
498 and the procedure being followed are clearly set out by the teacher running the practical. Students carry out
499 the practical, with assistance from demonstrators and analyse the data during the session.

500

501 However, there is scope for this practical to become more student-centred and to be used at a higher inquiry
502 level by making a number of modifications to the protocol. These could include giving the students less rigid
503 instructions on how and what haemoglobin standard solutions to produce and allowing them to make a wider
504 range of standard solutions to allow more accuracy in estimating the final degree of haemolysis. They could
505 also be asked to predict the impact of the different solutions on the haematocrit and haemoglobin
506 concentration and subsequently test these predictions. More sophisticated techniques could also be used to

507 measure haemolysis in the supernatant such as spectrophotometry and using light microscopy to visualise
508 the red blood cells after they have undergone crenation or haemolysis as opposed to the estimates made in
509 this experiment by eye.

510

511 This practical could be incorporated into a number of different biomedical programmes, from
512 Biology/Physiology/Biochemistry honours programmes to aid in the understanding of the fundamental
513 concepts of cell transport and membrane structure as well as developing vital scientific skills including
514 handling blood and performing serial dilutions. Professional disciplines such as medicine and veterinary
515 science would also benefit from this practical to further explore the concepts of osmotic fragility and the
516 administration of intravenous fluids and the clinical implications that these can have.

517

518 The concept of osmotic fragility could be explored further by using a series of hypotonic solutions and
519 recording % haemolysis. From these data an osmotic fragility curve could be plotted to explore the internal
520 pressures exerted on the cell membrane when water diffuses into a cell. Students could further consider how
521 the shape of red blood cells e.g. sickle cells may affect the osmotic fragility of red blood cells (8) and how
522 haemolytic diseases such as thalassemia and hereditary spherocytosis are a result of changes in osmotic
523 fragility both by extending this practical and the use of further resources (12).

524

525 Further examples of the differences between tonicity and osmolarity and the effect of permeable solutes can
526 be used, for instance with the addition of glucose, a particularly clinically relevant solute with regards to
527 intravenous administration of fluids. The clinical application of an understanding of this concept can be
528 emphasised including those surrounding patient safety (17).

529

530 **ADDITIONAL RESOURCES**

531 For additional information on this topic, any undergraduate level physiology textbook should provide
532 relevant background information required to understand the theory this practical is based upon.

533

534 **ACKNOWLEDGEMENTS**

535 We would like to thank Mr. Dave Gee, Senior Physiology Teaching Laboratory Technician and his technical
536 team for the invaluable help they provide in preparing this practical and for their assistance with this article.

537 We acknowledge the contribution of Professor Graham Mitchell for the initial design of this experiment.

538

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